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PROVISIONAL APPLICATION FOR PATENT COVER SHEET

This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

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[XX] *Additional inventors are being named on the* 1 *separately numbered sheets attached hereto*

TITLE OF THE INVENTION (500 characters max)

Use of Pleiotrophin in the Diagnosis, Treatment and Prevention of Disease

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ENCLOSED APPLICATION PARTS *(check all that apply)*

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- [ ] Drawing(s) *Number of Sheets*
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- [ ] Applicant claims small entity status. See 37 CFR 1.27.
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Respectfully submitted,

SIGNATURE _____ Date 7/15/03

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REGISTRATION NO. *(if appropriate)* 44,869

Docket Number: 304666

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USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

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Docket Number	81476-304666

INVENTOR(S)/APPLICANT(S)		
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Number 2 of 2

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APPLICATION FOR A PROVISIONAL
UNITED STATES PATENT
IN THE NAMES OF

BEHROOZ SHARIFI AND PREDIMAN K. SHAH

FOR

USE OF PLEIOTROPHIN IN THE DIAGNOSIS, TREATMENT AND PREVENTION OF DISEASE

ASSIGNED TO

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USE OF PLEIOTROPHIN IN THE DIAGNOSIS, TREATMENT AND PREVENTION OF DISEASE

FIELD OF THE INVENTION

The invention relates to the diagnosis, treatment and prevention of diseases in which neovascularization is a component of disease pathology as well as diseases in which the promotion of neovascularization is desirable. More specifically, the invention relates to neovascularization related to the effects of pleiotrophin.

DESCRIPTION OF THE INVENTION

The invention is based on the inventors' surprising discovery that pleiotrophin ("PTN") is related to neovascularization. While not wishing to be bound by any particular theory, it is believed that PTN is a plastogenic factor at least partially responsible for the transdifferentiation of macrophages and monocytes into endothelial cells. In fact, the inventors have surprisingly discovered that endothelial cells required for blood vessel formation need not originate from either pre-existing endothelial cells or their precursors; rather they may be generated *de novo* from other cell types. The various embodiments of the present invention set forth in greater detail, below, are predicated on this finding.

In one embodiment of the present invention, methods are provided to inhibit neovascularization by inhibiting the activity of PTN or its effects. This may be particularly advantageous in the treatment or prevention of diseases in which neovascularization is a component of disease pathology. Such diseases may include, by way of non-limiting example, cancer (*e.g.*, tumor growth and metastasis), diabetic retinopathy and rheumatoid arthritis.

Conversely, in another embodiment of the present invention, methods are provided to promote neovascularization by enhancing/promoting the activity of PTN or its effects. This may be particularly advantageous in the treatment of diseases such as, but in no way limited to, ischemia and the development of ischemic tissue. Inhibition of PTN may also be advantageous in the treatment of vascular disease, because it is believed to play a role in plaque development through the endothelial cells that reside within the plaque structure.

There are many ways in which one may inhibit the activity or effects of PTN, which will be readily apparent to one of skill in the art. By way of example, one may administer a drug or

peptide that blocks the action of PTN through its cell signaling cascade (*e.g.*, by blocking PTN itself or by blocking its receptor). Alternatively, the action or effects of PTN may be inhibited with synthetic molecules, RNA-based technology or ribozymes. In one particular embodiment, one may employ interference RNA ("RNAi") technology to inhibit the genetic activity of PTN.

There are also many ways in which one may upregulate, enhance or promote the activity of PTN. For example, one may employ any of a host of gene therapeutic techniques to cause or promote expression of the PTN gene *in vivo*. Such gene therapeutic approaches may be implemented with a vector, but this is not required. Alternatively, small molecules, peptides or other drugs that simulate the function of PTN may be administered in lieu of a promoter of PTN itself. Furthermore, by identifying the various components in the PTN cell signaling cascade, one may alternatively enhance that cascade by conventional methods, such as by administering a compound that is present in the cascade to increase signaling or by reducing the levels of a compound that stifles or otherwise reduces the effects of PTN through the cascade. In yet a further embodiment, one may administer an antibody against PTN and "immunize" an individual against its effects.

The delivery of any of the compounds or other products of the present invention may be performed locally or systemically, depending on the nature of the disease sought to be prevented or treated and other factors that will be readily apparent to one of ordinary skill in the art. In a still further embodiment of the present invention, PTN can be promoted or upregulated through non-gene cell therapy. In this embodiment, PTN may be overexpressed in, for example, a stem cell or a dendritic cell. The cell may then be implanted in a subject where it becomes part of the local tissue (*e.g.*, blood vessel).

EXAMPLE 1

New findings about the origin of cells and their regulators that contribute to tissue vascularization are likely to have implications for tumor growth /suppression and therapeutic angiogenesis for ischemic states. Here we report that pleiotrophin (PTN), an angiogenic factor, induces transdifferentiation of monocytic cells into endothelial cells. Using multiple independent methodologies, we show that monocytic cells exposed to TNF-α or IL-1β express PTN. Using a retroviral vector harboring PTN sense strand to infect cells, stable expression of PTN in monocytic cell lines led to their transdifferentiation into endothelial cells, as measured by the expression of widely used multiple endothelial cell markers that are expressed in mature endothelial cells. The expression levels of these markers were similar to that of human endothelial cells. *In vitro* studies showed that the PTN-infected cells can organize into vessel-like structure in three-dimensional culture. Injection of mouse monocytic cells stably expressing PTN into chick embryos showed that they can incorporate as endothelial cells and contribute to the developing vasculature in vivo. Our data show that PTN may contribute to neovascularization as a plastogenic/vasculogenic factor. This mechanism could have considerable consequences for anti-angiogenesis therapy for tumors and therapeutic vascularization of ischemic tissue.

Pathologic neovascularization accompanies chronic inflammation in psoriasis, synovial pannus formation in rheumatoid arthritis, atherosclerosis, and chronic granulomatous diseases (*1*). Several types of leukocytes, including macrophages, T cells, and mast cells, have been shown to activate immune–induced neovascularization (*2, 3*). Mononuclear phagocytes are recruited from peripheral blood to the sites of tissue injury where they undergo progressive differentiation to tissue macrophages that exert a paracrine effect to promote angiogenesis by releasing a myriad of angiogenic factors such as VEGF, PIGF, PDGF, bFGF, EGF, TGFβ and various interleukins and proteinases (*4*). These angiogenic factors induce recruitment and proliferation of endothelial cells from two sources: local residing endothelial cells, or recruitment of circulating endothelial progenitor cells. The first scenario is consistent with the classic paradigm of angiogenesis. Endothelial progenitor cells are circulating primitive bone marrow cells characterized by the expression of endothelial cell markers (*5*). Nevertheless, the mechanism of inflammation-induced angiogenesis and factors that regulate this event are poorly defined.

We have recently found that pleiotrophin (PTN) is strongly expressed in the atherosclerosis–prone coronary artery, but not in the atherosclerosis-resistant internal mammary arteries (*6*). PTN is a secreted protein that induces proliferation of various cell types, promotes angiogenesis, stimulates neurite outgrowth from cultured neurons, and induces cell migration (*7*). PTN is normally expressed during embryogenesis, but rarely in healthy adult tissues (*8*). However, PTN is re-expressed in some human tumors including breast cancer (*9*), pancreatic cancer (*10*), melanoma (*11*), meningioma (*12*) and neuroblastoma (*13*), and glioma (*14, 15*). Tumor cells transfected with dominant negative or ribozyme-targeted PTN and transplanted into nude mice yielded reduced tumor growth due to impaired angiogenesis (*9-11*) whereas breast carcinoma cells transformed by PTN develop into highly vascularized, aggressive tumors when implanted into nude mice (*16*). It is thought that PTN induces angiogenesis by stimulating endothelial cell proliferation and migration (*17*). We now demonstrate that PTN may induce neovascularization by an entirely different mechanism, transdifferentiation of monocytes into endothelial cells.

Since activated macrophages are considered the main source of angiogenic factors (*18*), we asked whether monocytic cells express PTN, and if so, what is the autocrine impact of the expression. Northern blot analysis showed that THP-1 monocytic cells do not express PTN (Fig. 1, lane 1); however, addition of either TNF-α (lane 2) or IL-1β (lane 3), pro-angiogenic/inflammatory factors, markedly up-regulated PTN mRNA levels. Similar results were obtained with RAW monocytic cells (not shown). In comparison, cultured human smooth muscle cells did not express PTN and were not affected by IL-1β or TNF-α treatment (Fig. 1, lanes 4-6). Similarly, cultured human dermal fibroblasts did not express PTN under these conditions (not shown). These data suggest that monocytic cells have the potential to express PTN in response to inflammatory stimuli. In addition, the data suggest that TNF-α or IL-1β activity in monocytic cells may be mediated, in part, by PTN.

Both TNF-α and IL-1β have pleiotrophic activity; therefore, they cannot be used to assess the autocrine activity of PTN in monocytes. Also, the recombinant PTN protein does not exhibit the full range of PTN activity (*19*). Therefore, we cloned full-length human PTN and generated stably transfected THP-1 and RAW cells in order to determine autocrine activity of PTN. Owing to plasticity of macrophages (*20, 21*) and pro-angiogenic activity of PTN (*16, 22*), we hypothesized that PTN promotes neovascularization by mediating transdifferentiation of

monocytes into endothelial cells. To test this, the expression of endothelial cell markers in PTN-infected monocytic cells was determined. The semi-quantitative RT-PCR analysis from three independent experiments is shown in Fig. 2A. THP-1 cells infected with PTN sense strand expressed vascular endothelial growth factor receptor-2 (Flk-1), Tie-2, vascular endothelial-cadherin (VE-cad), platelet endothelial cell adhesion molecule-1 (PECAM-1), endothelial nitric oxide synthase (eNOS), the von Willebrand factor (vWf), CD34, and stem/progenitor-cell markers AC133 ((lane 9), similar to that of positive control human coronary artery endothelial cells (lane 6). In contrast, these endothelial cell markers were not detected in either THP-1 cells infected with PTN anti-sense strand (lane 10) or GFP control vector (lane 11). Similarly, the expression of these markers was not detected in uninfected mouse monocytic RAW cells (lane 1), human monocytic THP-1 cells (lane 2), and human promonocytic leukemic U937 cells (lane 3). Further, endothelial cell markers were not expressed in negative control non-monocytic cells, such as NIH 3T3 cells (lane 4), human smooth muscle cells (lane 5), RPMI 8226 B lymphocyte plasmacytoma cell line (lane 7), and human skin fibroblasts (lane 8). The weak expression of FLK-1 in smooth muscle cells (lane 5) is consistent with the expression of this endothelial cell marker in human smooth muscle cells (*23*). The expression pattern of the endothelial cell markers in PTN-infected RAW cells was similar to PTN-infected THP-1 cells (not shown). Collectively, these data demonstrate that THP-1 and RAW monocytic cells do not express endothelial cell markers; however, PTN expression leads to expression of these markers. In addition, since the number of PCR cycles for each set of primers was chosen to be in the linear range of the amplification, these data show that the expression levels of endothelial cell markers are similar to those of positive control endothelial cells.

Recent studies have suggested that bone-marrow derived endothelial, hematopoietic stem and progenitor stem cells contribute to tissue vascularization during postnatal vasculogenesis (*24*). The monocytic cell lines that we used are established cell lines with known monocytic cell characteristics, suggesting that they do not have multipotent cell characteristics. The results of RT-PCR studies described above support this and show that the uninfected monocytic cells do not express either hematopoietic stem cell markers (Tie-2) or endothelial progenitor markers (Tie-2, CD34, and AC133) (*25*). In addition, uninfected or retroviral-infected mouse RAW cells did not express PECAM-1, the definitive marker of mice embryonic endothelial cells (*26*). Further, uninfected or retroviral-infected THP-1 cells did not express FLK-1, a marker of

undifferentiated, pluripotent endothelial cells (*27, 28*). However, to further investigate the pluripotency potential of the monocytic cells, we examined the expression of zinc finger transcription factors known to be expressed in endothelial cells such as GATA-2 and GATA-3 (*29-32*). Semi-quantitative PCR analysis (Fig. 2B) showed that THP-1 cells infected with PTN sense strand (lane 9) expressed both GATA-2 and GATA-3 transcription factors and that the expression levels of the factors are similar to control human endothelial cells (lane 6). In contrast, uninfected monocytic RAW, U937, and THP-1 cells (lanes 1-3) as well as THP-1 cells infected with either PTN anti-sense strand (lane 10) or GFP control vector (lane 11) did not express the transcription factors. In addition, non-monocytic NIH 3T3 cells (lane 4), smooth muscle cells (lane 5), RPMI 8226 B lymphocyte plasmacytoma cells (lane 7), and human skin fibroblasts (lane 8) did not express GATA-2 and GATA-3. In addition, we investigated the expression of Oct-4, a transcription factor that is required for the regulation of cell fate in the early embryo and is considered a marker of a cell's pluripotency (*33, 34*). PCR analysis revealed that none of the monocytic cells examined expressed Oct-4 (Fig. 2B), suggesting that they do not express a multipotent phenotype. Similarly, control non-monocytic cells such as smooth muscle cells, endothelial cells, and human fibroblasts did not express Oct-4. Interestingly, NIH 3T3 cells (lane 4) and RPMI 8226 B lymphocyte plasmacytoma cell line (lane 7) expressed Oct-4 suggesting that they have characteristics of immature cells. The immature nature of 3T3 cells is further supported by the expression of AC133 (panel A). Taken together with the data in Fig. 2A, we concluded that the monocytic cells do not have characteristics of pluripotent cells or endothelial progenitor cells.

Although the semi-quantitative RT-PCR showed that the expression level of endothelial cell markers in THP-1 and RAW cells infected with PTN sense strand is similar to the positive control endothelial cells, we used real-time PCR to accurately assess the expression levels of selected endothelial cell markers in the infected cells. Using primers specific for mature endothelial cells such as VE-cadherin, vWf, and PECAM-1, we found that the expression levels of these endothelial cell markers in THP-1 cells infected with PTN sense strand are similar to that of positive control endothelial cells but not in cells infected with the GFP control vector (Fig. 3). Similar results were obtained with Tie-2, and VE-cadherin (Fig. 3). GAPDH amplification was used as an internal standard. These results confirm the semi-quantitative RT-PCR data and show that the expression level of endothelial cell markers in PTN sense strand-

infected THP-1 cells is similar to the positive control endothelial cells, suggesting that they may be biologically relevant.

Having established expression of endothelial cell markers in the infected cells, we studied the distribution of VE-cadherin adhesion molecules by immunostaining in THP-1 cells infected with PTN sense strand (Fig. 4A). VE-cadherin positive cells appear to present a correct organization of adherent-type junctions, a class of cell adhesions characterized by their interaction with the actin microfilament system (*35*). No such expression was detected in THP-1 cells transduced with GFP control vector (Fig. 4A). Further analysis of cells with various endothelial specific proteins indicated that these cells are endothelial-like cells, expressing FLK-1 and Tie-2, membrane-bound tyrosine kinases, at the cell surface and vWf in large granules dispersed throughout the cytoplasm (not shown).

To determine the topographical relationship between distribution of transcription factors and endothelial cell markers in the infected cells, we performed double staining of PTN-infected THP-1 cells with anti-human GATA-2 rabbit polyclonal antibody and anti-human VE-cadherin mouse monoclonal antibodies. A representative confocal image of THP-1 cells infected with PTN sense strand is shown in Fig. 4B. Consistent with the light microscopy results, we found strong expression of VE-cadherin on the surface of PTN-infected cells (red color) while GATA-2 expression was concentrated in the nucleus (blue color). The overlay showed the co-expression of the two endothelial cell markers in THP-1 cells infected with PTN sense strand. No VE-cadherin or GATA-2 staining were detected in THP-1 cells infected with the control vector (not shown).

To further confirm an endothelial-like phenotype of THP-1 cells infected with PTN sense strand, we assayed them for the expression of Tie-2. The infected THP-1 cells were incubated with fluorescent-labeled antibodies and analyzed by fluorescence-activated cell sorter (FACS, Becton Dickinson). FACS analysis revealed that 65% of PTN-infected THP-1 cells expressed Tie-2, as compared to 15% of cells infected with GFP control retroviral vector (Fig. 5A). In addition to Tie-2, we investigated the expression of integrin in the infected cells, as neovascularization depends on specific molecular interactions between vascular cells and components of the extracellular matrix. Of the wide spectrum of integrin subunit combinations that are expressed on the surface of cells, integrin $\alpha_v\beta_3$ has been identified as having an especially interesting expression pattern among vascular cells during angiogenesis and vascular

remodeling (*36*). Integrin $\alpha_v\beta_3$ is a receptor for a wide variety of ECM ligands with an exposed RGD sequence, including vitronectin, fibronectin, fibrinogen, thrombospondin, proteolyzed collagen, von Willebrand factor, and osteopontin (*37*). FACS analysis of PTN-infected THP-1 cells showed that 80% of cells expressed $\alpha_v\beta_3$ integrin, as compared to 1% of THP-1 cells infected with GFP control retrovirus vector (Fig. 5B). FACS analysis of positive control human coronary artery endothelial cells showed 80% of cells expressed $\alpha_v\beta_3$ integrin and omission of anti-$\alpha_v\beta_3$ antibody reduced positivity to 4% (Fig. 5C). These data demonstrate that PTN induces the de novo expression of $\alpha_v\beta_3$ in vitro, suggesting that it could play a role in the initiation of PTN-mediated neovascularization. The up-regulation of $\alpha_v\beta_3$ synthesis in the replicating PTN-infected cells is consistent with the expression pattern of this integrin in vascular endothelium, as $\alpha_v\beta_3$ integrin is poorly expressed on quiescent vascular endothelium and is expressed at high levels only after growth-factor stimulation of endothelium ????? (15).

To study this vascularization-like process further, we investigated the ability of the transduced THP-1 and RAW cells to form tubular structures. PTN- and GFP-transduced cells were cultured on three-dimensional fibrin matrices. Uninfected THP-1 and RAW cells were used as negative controls and human endothelial cells were used as a positive control. After 3 days in culture, cells infected with PTN sense strand invaded the fibrin matrix and started to form network-like structures in the three-dimensional gel, similar to positive control endothelial cells (not shown). In contrast, uninfected THP-1 and RAW cells as well as cells infected with the control GFP vector remained on top of the fibrin matrix and no network-like structure could be observed (not shown). These data demonstrate that infection of monocytic cells with PTN sense strand confers ability to the cells to rearrange in the fibrin gel with extended cytoplasm and interact with surrounding cells, similar to endothelial cells. These data correlate with the RT-PCR and immunostaining data that demonstrate up-regulation of RNA and protein levels of the endothelial genes in monocytic cells infected with PTN sense strand.

Having established expression of endothelial cell markers in PTN-infected monocytic cells in vitro, we asked about the in vivo differentiation potential of these cells. RAW cells stably expressing human PTN sense strand were injected intracardially into stage 16-17 chick embryos and we traced them by immunostaining. Most of the immunopositivity appeared along the vessels in the head, eyes, heart, and intersomitic reagion (Fig. 6A), and in some cases forming a network structure 2-3 days post-injection (Fig. 6B). However, injection of RAW cells

expressing GFP control vector did not stain (Fig. 6C), and in some cases faint GFP staining was found around the amniotic cavity. This indicates that PTN-infected cells differentiate to endothelial-like cells and contribute to the vascular component. Staining of serial sections with anti-Tie-2 antibody showed a similar distribution pattern (not shown).

We have provided several independent lines of evidence suggesting that the angiogenic protein PTN induces transdifferentiation of monocytic cells into endothelial-like cells. The induced endothelial cell markers such as VE-cadherin, PECAM-1, vWf, eNOs, and $\alpha_v\beta_3$ are generally expressed in mature endothelial cells, suggesting that the transdifferentiated monocytioc cells have mature endothelial-like cell phenotype. We found that the expression of endothelial cell markers is associated with the ability of PTN-infected monocytic cells to form tubular structure in vitro and contribute to vasculogenesis in vivo, suggesting that the expression of endothelial markers are functionally significant and the PTN-infected cells may function in a vascular-related capacity.

Growth and stabilization of functional vessel in adult tissues may require joint effects of multiple factors that target different cell populations. Past studies have considered PTN as an angiogenic factor based on its ability to stimulate endothelial cell proliferation. We propose a new role for PTN, transdifferentiation of monocytic cells into endothelial cells. The mechanism underlying the transdifferentiation of monocytic cells into endothelial-like cells by PTN may be complex. We do not know whether this plastogenic/vasculogenic activity of PTN is restricted to monocytic cells or it exerts similar autocrine effect on other cell types, particularly tumor cells. Although PTN is highly expressed during embryogenesis, its expression in adult tissue is restricted to brain. It is, however, re-expressed in tumors and in inflamed tissues. PTN is generally produced in highly vascularized tumors such as glioma, where it is thought that it promotes neovascularization through angiogenesis (*15*). In addition to angiogenesis and postnatal vasculogenesis pathways, tumor cells can assume endothelial-like cell characteristics, vascular mimicry. For example, the expression of VEGF, Flk-1, and Flt-1 (VEGF-R1) has been detected in a variety of human tumor cell lines of nonendothelial origin (*38*). These included melanoma, ovarian, pancreatic, prostate carcinomas, breast cancer, and Kaposi's sarcoma. Using the nonaggressive and aggressive breast cancer cell lines, it was shown that aggressive breast cancer cells express Tie-2 and CD31 (*39*). Similarly, Tie-2 and angiopoietins expression has been detected in tumor cells of Kaposi's sarcoma (Brown, LF, Dezube, BJ, Tognazzi, K,

Dvorak, HF, & Yancopoulos, GD: Expression of tie1, tie2, and angiopoietins 1, 2, and 4 in Kaposi's sarcoma and cutaneous angiosarcoma. Am J Pathol 2000, 156:2179). Microarray analysis of highly aggressive and poorly aggressive human cutaneous melanoma cell lines showed that VE-cadherin and Tie-2, was exclusively expressed by highly aggressive melanoma cells and was undetectable in the poorly aggressive tumor cells (*40*). Factors that contribute to this phenotypic modulation of tumor cells are not known; however, PTN is expressed by the tumors that exhibit this modulation such as melanoma cells (*11*), pancreatic cancer (*10*), prostate cancer (*41*), and breast tumor (*42*). Based on our data, it is tempting to speculate that PTN may be involved in the phenotypic conversion of these cancer cells into endothelial-like cells, enabling them to function in a vascular-related capacity.

In addition to tumor vascularization, PTN may be involved in inflammation-induced neovascularization, as it is expressed in the synovial fluid of patients with osteoarthritis (*43*) and rheumatoid arthritis (*44*). In these diseases, PTN expression is up-regulated in synovial fibroblasts by proinflammatory cytokines (*44*). We also found that PTN mRNA is markedly up-regulated by TNF-α and IL-1β in macrophages. Therefore, monocytes/macrophages in synovial fluid may be exposed to high levels of PTN produced by both fibroblasts and monocytes/macrophages. Since PTN promotes proliferation of monocytic cells (*44, 45*), it is possible that the expression of PTN in synovial fluid generate a feedback mechanism that leads to proliferation of monocytes followed by their transdifferentiation into endothelial-like cells. This may exacerbates tissue inflammation.

Past studies have used primary population of cells, with varying degree of cell impurity, to investigate differentiation of progenitor cells. Considering that as few as 100 hematopoietic stem cells are capable of repopulating hematopoietic tissue of lethally irradiated mice (*46*), preparations containing a few thousand or even a few cell contaminants complicate the interpretation of data on the contribution of endothelial progenitor cells (*5, 47-49*) or mononuclear cells (*50-52*) to angiogenesis or postnatal vasculogenesis. Since we have used homogeneous population of clonal cells, the interpretation of our transdifferentiation data does not have this limitation.

We have identified a novel mechanism underlying contribution of PTN and monocytic cells to neovascularization. We offer new evidence that monocytic cells transdifferentiate into endothelial cells by PTN, an angiogenic factor that is produced by tumor cells. Although the

underlying molecular mechanisms are not understood, identification of PTN as a plastogenic/vasculogenic factor and knowledge of the origin of cells lining new blood vessels highlight dual functions of monocytic cells in neovascularization: their direct role as a source of endothelial-like cells, in addition to their traditional role as a source of angiogenic and growth factors. Our finding offers new targets to prevent neovascularization of tumors or to promote blood circulation in ischemic tissues.

Expression of PTN by monocytic cell lines. Human THP-1 and mouse RAW monocytic cells were obtained from ATCC and cultured in RPMI/10% fetal bovine serum, as recommended. In some studies, the media was supplemented with 50 ng/ml TNF-α or 20 ng/ml IL-1β for 6 h. For comparison, we used human coronary artery smooth muscle cells and human dermal fibroblasts (both obtained from Cell Applications, Inc.) and cultured according to their instructions. RNA isolation and Northern blot were performed essentially as described (*53*). Briefly, cultured cells were lysed with guanidine thiocyanate and RNA was isolated by centrifugation through a cesium chloride cushion. The RNA was quantified and 10 μg/lane was analyzed by electrophoresis followed by blotting. The blot was probed with the PTN cDNA probe (top panel). To control for loading, the blot was re-probed with β-actin (lower panel).

Endothelial gene expression in monocytic and non-monocytic cells by RT-PCR analysis. (*A*) RNA was isolated and separated by agarose gel electrophoresis. Mouse RAW (lane 1), human THP-1 (lane 2), human U937 (lane 3) monocytic cells. The non-monocytic cells, NIH 3T3 cells (lane 4), human coronary artery smooth muscle cells (lane 5), RPMI 8226 B lymphocyte plasmacytoma cell line (lane 7), and human dermal fibroblasts (lane 8) were used as negative controls and human coronary artery endothelial cells (lane 6, obtained from Cell Applications, Inc.) were used as a positive control. Otherwise indicated, cells were obtained from ATCC and cultured, as recommended. In addition, RNA was isolated from THP1 cells infected with bicistronic retrovirus vector harboring either PTN sense strand (lane 9), PTN anti-sense strand (lane 10), or green fluorescence protein (lane 11). The RNA was subjected to RT-PCR analysis, using specific primers for vascular endothelial growth factor receptor-2 (Flk-1)(*54*), tyrosine kinase receptor Tie-2 (*55*), vascular endothelial-cadherin (VE-cad)(*56*), PECAM-1(*57*), endothelial nitric oxide synthase (eNOS)(*58*), the von Willebrand factor (vWf)(*58*), CD34 (*59*), and AC133/CD133, a cell surface marker of vascular/hematopoietic stem and progenitor cells (*60*). To ensure semi-quantitative results of the RT-PCR assays, the number

of PCR cycles for each set of primers was checked to be in the linear range of the amplification. In addition, all RNA samples were adjusted to yield equal amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard. The amplified products were separated on 1.2% agarose gels and stained with ethidium bromide. (*B*) Expression of transcription factors in monocytic and non-monocytic cells. RNA isolated from cells was subjected to PCR analysis using primers specific for GATA-2, GATA-3, and Oct-4 transcription factors. The amplified PCR products were analyzed by agarose gel as described in panel A. Cloning of PTN, preparation of bicistronic retrovirus, primer sequences, reaction conditions, and optimal cycle numbers are published as supporting information on the Science web site.

Real-time PCR analysis of VE-cadherin, vWf, and PECAM-1. PCR was performed on a Perkin-Elmer/Applied Biosystem 7700 Prism instrument using cDNA reverse-transcribed from three different preparations of RNA isolated from: THP-1 cells infected with PTN sense strand (blue, dark blue, magenta), human coronary artery endothelial cells (dark green, deep blue, and light red), and THP-1 cells infected with retrovirus harboring GFP gene (yellow, light green, and dark red). GAPDH was used as an internal reference control.

Expression of endothelial cell markers in PTN-infected THP-1 cells. (*A*) PTN- or GFP-infected THP1 cells were cultured on gelatin-coated cover-slips in the presence of 10% (vol/vol) FBS. After attachment to the cover slips, cells were fixed with methanol for 5 min at 20°C or with 3% (wt/vol) paraformaldehyde at room temperature and stained for 30 min with the relevant primary antibodies: anti-human FLK-1 mouse monoclonal (Santa Cruz, California), anti-human VE-cadherin mouse monoclonal (Santa Cruz, California), or anti-human Tie-2 rabbit polyclonal (Santa Cruz, California). The secondary antibodies were HRP-labeled goat anti-mouse or goat anti-rabbit antibodies (both from Santa-Cruz California). The antibodies were used at the dilutions recommended by Santa Cruz. B) For confocal microscopy, the secondary antibodies (Alexa Fluor 633 goat anti-rabbit antibody (blue) and Alexa Fluor 568 goat anti-mouse antibody (red)) were used at 1:500 dilutions, as recommended by Molecular Probe. After the indirect immunolabeling, cells were mounted in Floromount-G (Southern Biotechnology) and were examined with either a conventional fluorescence microscope (Nikon) or Zeiss LSM 510 confocal microscope.

Flow cytometry evaluation of infected THP-1 and endothelial cells. THP-1 cells infected with bicistronic retrovirus harboring either PTN+GFP or GFP were examined for the

expression of Tie-2 and $\alpha_v\beta_3$. Flow cytometry profiles were obtained by incubating the cells with a 1:100 dilution of either anti-human Tie-2 rabbit polyclonal antibody (Santa Cruz, California) or anti-human $\alpha_v\beta_3$ mouse antibody (Chemicon Co) for 30 min on ice. After washing, cells were incubated with either 1:500 dilution of PE-labeled anti-rabbit antibody (Molecular Probes) or PE labeled anti-mouse antibody (Sigma) for 15 min on ice. After washing, cells were fixed with 1% paraformaldehyde and analyzed by FACS. In addition, human coronary artery endothelial cells were used as a positive control. In all experiments, omission of the primary antibody was used to control for non-specific staining. Tick marks on the x-axis designate logarithmic increments in fluorescent intensity. Panel A, Tie-2 expression of THP-1 cells infected with GFP (top panel) or PTN-infected cells (lower panel). Panel B, $\alpha_v\beta_3$, expression of cells infected with GFP (top panel) or PTN-infected cells (lower panel). Panel C is FACS analysis of $\alpha_v\beta_3$, expression by positive control endothelial cells in the absence (top panel) or presence of anti-$\alpha_v\beta_3$ antibody.

Contribution of PTN-infected RAW cells to vascular formation in vivo. Mouse monocytic RAW cells (1-2 $X10^5$ cells in 2-4 µl) infected with bicistronic vector that expresses PTN sense strand, in addition to GFP, were injected into the hearts of stage 16-17 chick embryos. Embryos were killed 2-3 days after injection, fixed, embedded in OTC, and frozen sections were cut and stained with anti-green fluorescent polyclonal antibody (Santa Cruz). We chose to stain the sections with anti-GFP antibody, rather than PTN, because PTN is expressed during embryogenesis (*61-65*). Panel A shows immunostaining (brown color) localized along blood vessels in the head, eyes, heart, and intersomitic region. Panel B shows a confocal image of the chicken embryo from panel A which exhibits the association of GFP signal with microvessels in the head region. Panel C shows the immunostaining of embryo injected with RAW cells expressing only GFP gene. In some cases, faint staining was detected around the amniotic cavity.

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EXAMPLE 2

We recently discovered unique gene expression profiles of coronary and mammary arteries. While coronary arteries are prone to develop angiogenic sprouting and consequently, develop atherosclerotic plaques, internal mammary arteries are remarkably resistant to plaque formation. Using suppressive subtraction hybridization, we have identified genes that are differentially expressed in the porcine coronary and mammary arteries. We found that coronary artery strongly expressed pleiotrophin (PTN), a pro-angiogenic gene, whereas mammary artery does not express this gene [Qin, 2003 #3127].

PTN is normally expressed during embryogenesis, but rarely in healthy adult tissue. In the brain, PTN is expressed during embryonic development (see Fig. 9) and its expression is down-regulated in the adult brain. However, it is re-expressed in regions with high levels of neovascular formation of glioblastomas and the adult ischemic brain. In addition, PTN is produced by other human tumors such as breast cancer, pancreatic cancer, melanoma, meningioma, and neuroblastoma. Tumor cells transfected with dominant negative or ribozyme-targeted PTN and transplanted into nude mice yielded reduced tumor growth due to impaired angiogenesis whereas breast carcinoma cells overexpressing PTN have a growth advantage.

It is thought that PTN promotes tumor vascularization by stimulating endothelial cell recruitment, proliferation, and angiogenesis. We offer evidence that PTN may promote tumor vascularization through an entirely different mechanism that is independent of angiogenesis. Based on our findings, we propose that PTN promotes tumor vascularization, in part, by inducing transdifferentiation of monocytes/macrophages into endothelial cells. This study is designed to expand and extend this proposal.

Aim 1) Does PTN induces transdifferentiation of glioma cells into endothelial-like cells?

We have found that monocytes/macrophages can be converted into endothelial-like cells when stably transduced with retrovirus harboring PTN sense strand, but not PTN anti-sense strand or GFP control vector. We would now like to expand on these observations by asking whether this plastogenic activity of PTN is limited to monocyte/macrophages or whether tumor cells, such as glioma, can be coaxed by PTN to alter their phenotype into endothelial-like cells. To address this question, we will transduce glioma cells with the retrovirus harboring PTN and determine the phenotypic characteristics of the infected cells.

Aim 2) What is the active domain of PTN?

This aim will extend the preliminary data and effort of aim1 by asking which segment or domain of the PTN molecule is responsible for its transdifferentiation activity. Mapping of the active domain of PTN that is required for transdifferentiation of monocytic cells, or possibly glioma cells (aim 1), will help us to understand the molecular basis of PTN signaling. Prior studies have utilized a series of constructed PTN mutant proteins to determine the domains required for the transformation activity [Zhang, 1999 #3187]. We will construct a series of PTN mutants and test their ability to transdifferentiate monocytic cells.

Aim 3) What is the downstream signaling in PTN-mediated transdifferentiation?

This aim will extend the efforts of aims 1 and 2 by investigating the PTN-induced signaling. Currently, nothing is known about PTN signaling in monocytes/macrophages. As an initial step to understand PTN signaling involved in transdifferentiation of monocytes/macrophages to endothelial cells, we will concentrate our effort on the mitogen-activate protein (MAP) kinase pathway for two reasons: 1] this pathway is known to be activated by PTN in bovine epithelial lens cells [Souttou, 1997 #3086], and 2] MAP kinase pathway is thought to be a key signaling pathway that have been implicated in the phenotypic outcome of endothelial cells and angiogenesis [Lee, 2000 #3213]. Activation of MAP kinase pathway has been investigated in a variety of macrophages including THP-1 cells [Huang, 1999 #3200;McGilvray, 1998 #3198;Hambleton, 1996 #3199].

Background and significance

Vascularization of tumors is a highly complex process that is regulated by a balance between pro- and anti-angiogenic molecules [Carmeliet, 2000 #3101]. Pro- and anti-angiogenic molecules can emanate from cancer cells, endothelial cells, inflammatory cells, stromal cells, blood and the extracellular matrix. Three models have been proposed to explain tumor vascularization: angiogenesis, postnatal vasculogenesis, and vascular mimicry.

Angiogenesis: According to this model, endothelial cells are derived from the pre-existing endothelium of blood vessels. Recruitment and proliferation of endothelial cells is regulated by angiogenic factors that emanate from tumor cells, infiltrating leukocytes, and stromal cells. Among the hematopoietic cells, macrophages are thought to be critical in promoting angiogenesis by: a] releasing potent angiogenic factors such as VEGF, bFGF, TGF-α,

TNF-α, and IL-8, which induce recruitment and proliferation of endothelial cells from pre-existing vessels [Brogi, 1993 #3099;Ramos, 1998 #3104;Seljelid, 1999 #3112], and b] modulating extracellular matrix remodeling required for new blood vessel formation through secretion of various metalloproteinases (see Ono [Ono, 1999 #3136] for review). Thus, macrophages influence every stage of neovascularization by a paracrine mechanism, i.e., releasing factors that promote recruitment and proliferation of endothelial cells.

Postnatal vasculogenesis: The angiogenic factors produced by tumor cells recruit endothelial cells from two sources: pre-existing mature endothelial cells and circulating endothelial progenitor cells. Mature endothelial cells can be recruited either from pre-existing blood vessels (angiogenesis) or from circulating endothelial cells. Normal adults have 2.6 ± 1.6 circulating endothelial cells per mm^3 of peripheral blood with most of these cells being quiescent and at least half being microvascular as defined by CD36 positivity [Solovey, 1997 #3058]. Circulating mature endothelial cells are detectable in pathological diseases marked by vascular injury conditions, such as sickle cell anemia, acute myocardial infarction, thrombotic thrombocytopenic purpura, and active cytomegalovirus infection [Solovey, 1997 #3058;Lefevre, 1993 #3059;Grefte, 1993 #3060;Hladovec, 1978 #3123;Hladovec, 1978 #3124].

The existence of circulating endothelial progenitor cells in adult humans as a characteristic feature of postnatal vasculogenesis has only recently been suggested. Asahara et al. [Asahara, 1997 #2897] isolated endothelial progenitor cells from adult human peripheral blood using magnetic bead selection of CD34+ hematopoietic cells. In vitro, majority of the primary adherent cells differentiated into spindle-shaped cells within 7-10 days of culture on fibronectin and expressed markers of endothelial cell characteristics. Kalka et al. [Kalka, 2000 #3163] used the primary adherence on fibronectin to isolate endothelial progenitor cells from total human peripheral blood mononuclear cells and also demonstrated the appearance of cells with an endothelial phenotype at a very high frequency after 7-10 days of culture. Animal models of ischemia and tumor growth demonstrated the contribution of endothelial progenitor cells to active neovascularization [Kalka, 2000 #3163;Asahara, 1999 #2898;Takahashi, 1999 #3067]. Shi et al. [Shi, 1998 #2904] and Nieda et al. [Nieda, 1997 #3164], using CD34+ cells at a much higher purity (>93%) than Asahara et al. [Asahara, 1997 #2897], observed adherent endothelial cell colonies. Since human peripheral blood was used as a stating point to isolate endothelial progenitor cells in these studies, the potential contribution of contaminating cells in

the interpretation of the data remains unclear. The experiments described above on the characterization of endothelial progenitor cells used cell preparations with 7% [Shi, 1998 #2904;Nieda, 1997 #3164] to 86% [Asahara, 1997 #2897] impurities, raising concerns about the origin of endothelial cells. Since endothelial progenitor cells have been isolated from the human peripheral blood mononuclear cell fraction containing varying degrees of monocytic cell contaminants that have a high capacity to adhere to extracellular matrix at the time of isolation, it is conceivable that monocytic cells, or its subpopulation, may be the source of endothelial progenitor cells. We offer evidence that support this notion (see preliminary data).

In addition to circulating mature and immature endothelial cells, transdifferentiation of bone marrow stem cells represents another source of endothelial cells. Several studies have suggested the existence of multipotent adult stem cells that have the potential to replenish several cell lineages in various tissues, even across the germ layer barrier [Orkin, 2000 #3053]. Adult hematopoietic cells are defined by their ability to self-renew while functionally repopulating the hematopoietic compartment for the lifetime of an individual. Like other tissue-specific stem cells, hematopoietic stem cells could retain plasticity capable of regenerating multiple cell types in non-hematopoietic tissues, including the endothelial cells. Multipotent adult mesenchymal stem cells also differentiate into many specialized cell types in culture and contribute to a wide range of developing tissues when injected into mouse blastocysts. When transplanted into adult mice, they engraft and differentiate into hematopoietic cells, epithelial cells and endothelial cells [Jiang, 2002 #3054].

Similar concerns about the role of cell contaminants have been raised in the interpretation of engraftment and differentiation of stem cells [Goodell, 2001 #3166]. While recent studies with purified hematopoietic stem cells indicate that, at least in some cases, stem cells or their progeny can transdifferentiate into nonhematopoietic cells, a definitive proof of transdifferentiation is still lacking, mainly due to cell populations, rather than a single cell, being used in the experiments. Preparations of a few thousand, or even few, purified hematopoietic stem cells could still include progenitors of other tissues as contaminants, raising the possibility that the grafts could be heterogeneous and therefore, the probability of two different types of cells contributing to two different lineages cannot be excluded. We offer evidence that human and mouse monocytic cell lines that represent homogeneous population of cloned cells can be induced to transdifferentiate into endothelial-like cells (see preliminary data).

Vascular mimicry: Some tumors are vascularized without significant angiogenesis, by forming vascular channels on their own through a non-endothelial cell process [Maniotis, 1999 #3168]. For example, 15% of blood vessels in xenografted and spontaneous human colon carcinomas are mosaic in nature [Chang, 2000 #3260]. It has been suggested that highly invasive primary and metastatic melanoma cells may generate vascular channels, lined externally by melanoma cells themselves, and facilitate tumor perfusion independent of angiogenesis [Folberg, 2000 #3169]. It is reported that tumor cells migrate toward existing host organ blood vessels in sites of metastases, or in vascularized organs such as the brain, to initiate blood vessel-dependent tumor growth as opposed to classic angiogenesis [Holash, 1999 #3170]. It is an open question whether these vessels result from cancer cells invading the vessel lumen, cancer cells mimicking endothelial cells, co-opted vessels or apoptosis of endothelial cells which exposes underlying cancer cells.

In summary, it is thought that tumor angiogenesis starts only when the neoplastic mass reaches 1 mm in diameter and when hypoxia occurs and that it is essentially mediated by angiogenic molecules elaborated by tumor cells and infiltrating leukocytes. Nevertheless, the mechanisms responsible for vascularization of in situ tumors and their remodeling are still poorly understood. Three models have been proposed to describe vascularization of tumors with differences based on the origin of blood vessel cells. The new capillary can be formed by endothelial cells derived from either pre-existing blood vessels (angiogenesis), or endothelial progenitor cells (postnatal vasculogenesis). Besides endothelial cells, blood vessel walls can be lined with cancer cells alone or a mosaic of cancer and endothelial cells (vascular mimicry). We offer evidence for the existence of a new model for capillary formation. This new model is based on the transdifferentiation of monocytes/macrophages, or possibly tumor cells, into endothelial cells that could subsequently contribute to tumor vascularization.

Preliminary Studies

Hypothesis:

Recent evidence suggests that the ability of immature cells to differentiate into different cell types is not an exclusive feature of progenitor cells. Rather, post-mitotic differentiated cells or differentiation-committed cells can also undergo transdifferentiation. For example, hepatic oval cells could be re-differentiated to hormone-producing pancreatic islet cells by culturing in

high-glucose medium [Yang, 2002 #3184]. Conversely, exposure of a pancreatic cell line to glucocorticoid resulted in their transdifferentiation to hepatic cells [Shen, 2000 #3135]. In a co-culture model, neurosphere-derived cells can differentiate into skeletal muscle cells in the presence of differentiating myoblasts [Rietze, 2001 #3185]. Finally, injection of 3T3-L1 preadipocyte cells into the peritoneal cavity of nude mice converted the cells into macrophages [Charriere, 2003 #3134]. These studies suggest that differentiation is an ongoing process that can be modified by key regulators. Factors that regulate a cell's plasticity remain largely unknown.

Since both endothelial and hematopoietic cell lineages share a common precursor during embryogenesis (hemeangioblasts) and committed cells are capable of switching their fate, we reasoned that monocytes could be converted into endothelial cells. Specifically, we hypothesize that angiogenic factors may promote neovascularization by inducing transdifferentiation of infiltrating monocytes into endothelial-like cells. A corollary hypothesis would hold that angiogenic factor may behave as a plastogenic factor.

We recently discovered a unique gene expression profile of coronary and mammary arteries. While coronary arteries are prone to develop angiogenic sprouting and consequently, develop atherosclerotic plaques, internal mammary arteries are remarkably resistant to plaque formation. We hypothesized that the normal coronary artery has an environment that is conducive to angiogenic processes. In contrast, the normal internal mammary artery milieu counters this event. We used suppressive subtraction hybridization (SSH) to generate reciprocal cDNA collections of representing mRNA specific to porcine coronary vs. porcine mammary arteries. We screened 1000 SSH cDNA clones by dot blot array and sequenced 600 of these showing the most marked differences in expression. Northern blot and in situ hybridization confirmed the differential gene expression pattern identified by the dot blot arrays.

We found that pleiotrophin (PTN) is strongly expressed in the pro-angiogenic coronary artery, but not in the angiogenic-resistant internal mammary arteries [Qin, 2003 #3127]. PTN is a 18-kDa protein that contains 24% basic residues (18% lysines), arranged mainly in two clusters at the N- and C-terminal regions, and five intra-chain disulfide bonds (for general discussions see Muramatsu [Muramatsu, 2002 #3021]). The molecule is organized in two β-sheet domains linked by a flexible linker with each of these domains having a heparin-binding site. At least one heparin-binding site is involved in the dimerization of this growth factor that is important for

PTN mitogenic activity since this activity on BEL cells is modulated by exogenous addition of glycosaminoglycans [Vacherot, 1999 #3259]. Furthermore, treatment of BEL cells with heparinase III abolished PTN mitogenic activity, which could be restored by the addition of soluble heparin.

PTN is mitogenic for various cell types, promotes angiogenesis, stimulates neurite outgrowth from cultured neurons, and induces cell migration. PTN is normally expressed during embryogenesis, but rarely in healthy adult tissues [Iwasaki, 1997 #3215]. However, PTN is produced by some human tumors including breast cancer [Zhang, 1997 #3179], pancreatic cancer [Weber, 2000 #3216], melanoma [Czubayko, 1996 #3178], meningioma [Mailleux, 1992 #3039] and neuroblastoma [Nakagawara, 1995 #3041]. Tumor cells transfected with dominant negative or ribozyme-targeted PTN and transplanted into nude mice yielded reduced tumor growth due to impaired angiogenesis [Czubayko, 1996 #3178;Zhang, 1997 #3179;Weber, 2000 #3216] whereas breast carcinoma cells overexpressing PTN have a growth advantage [Choudhuri, 1997 #3193]. Thus, various tumor cells produce the angiogenic factor PTN.

PTN expression in the brain occurs mainly during embryonic and early postnatal periods, but not in the adult brain [Bloch, 1992 #3220]. PTN is re-expressed in glioblastoma and in adult rat brain after acute ischemia. In high-grade gliomas, PTN mRNA or protein is detectable and its expression is confined to proliferating cells in situ [Mentlein, 2002 #3219]. It is thought that PTN produced by gliomas contributes to their malignancy by targeting endothelial cells [Mentlein, 2002 #3219]. In the ischemic brain, PTN expression is concentrated within areas of exuberant neovasculature that formed at the margins of the infarct and in macrophages around the newly formed vessels [Takeda, 1995 #3080;Yeh, 1998 #3042]. It has been suggested that PTN produced by macrophages promote brain angiogenesis by stimulating recruitment of endothelial cells [Takeda, 1995 #3080;Yeh, 1998 #3042].

Macrophages are thought to be critical in promoting angiogenesis by releasing potent angiogenic factors that stimulate recruitment and proliferation of endothelial cells from preexisting vessels [Brogi, 1993 #3099;Ramos, 1998 #3104;Seljelid, 1999 #3112] and by modulating extracellular matrix remodeling required for new blood vessel formation through secretion of various metalloproteinases (see Ono [Ono, 1999 #3136] for review). Thus, macrophages influence every stage of neovascularization by a paracrine mechanism, i.e., releasing factors that promote recruitment and proliferation of endothelial cells.

In addition to the effects on neighboring cells, we hypothesized that angiogenic factors produced by macrophages affect activity of monocytes/macrophages in an autocrine fashion. Since, PTN expression is concentrated within areas of exuberant neovasculature and co-localized with macrophages in the ischemic brain [Takeda, 1995 #3080;Yeh, 1998 #3042], we asked whether macrophages express PTN, and if so, what effects does this expression have on macrophage activity. There is no published report on the activity of PTN on macrophages.

Expression of PTN by monocytic cells

In preliminary experiments, we first determined whether PTN is expressed by monocytes/macrophages. In addition, since activated tumor-associated macrophages express TNF-α [Ono, 1999 #3136], a known potent angiogenic factor, we asked whether TNF-α regulates expression of PTN in macrophages. We treated exponentially growing cultured human THP-1 and mouse RAW monocytic cells (obtained from ATCC) with TNF-α (in RPMI/10% serum) for 6 h. Control cells were maintained in RPMI/10% bovine serum. In addition, we used exponentially growing human fibroblastic cells (DMEM/10% serum) for comparison. RNA isolation and Northern blot was performed essentially as we described [LaFleur, 1994 #462]. Briefly, cultured cells were lysed with guanidine thiocyanate and RNA was isolated by centrifugation through a cesium chloride cushion. The RNA was quantified and 10 μg/lane was analyzed by electrophoresis followed by blotting. The blot was probed with the PTN cDNA probe (top panel). To control for loading, the blot was re-probed with β-actin (lower panel).

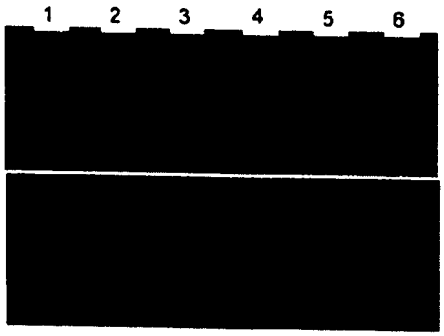


Fig. 1. Expression of PTN by activated monocytic cells.

Northern blot analysis revealed that untreated, exponentially growing cultured human THP-1 (Fig. 1, right panel, lane 1) and mouse RAW (lane 4) cells do not express PTN mRNA. Addition of 10 ng/ml of TNF-α markedly up-regulated expression of PTN in human THP-1 cells (lane 2), and in mouse RAW cells (lane 3). Exponentially growing cultured human fibroblasts did not express PTN (lane 5) and addition of TNF-α had no effect (lane 6). These data demonstrate that: 1] the expression of PTN in macrophages is regulated by TNF-α and 2] the signaling events activated by TNF-α in the expression of PTN is conserved in human and mouse. These data suggest that TNF-α activity in

macrophages may be mediated, in part, by PTN.

To further test our hypothesis of the role of PTN in macrophage-mediated angiogenesis, we have three options: 1] Add TNF-α to cells and examine the effect. This would not be a good idea because TNF-α is a pleiotrophic factor that has many known and unknown effects on macrophages, which would complicate interpretation of the data. 2] Add recombinant PTN to monocytic cells and evaluate the outcome. This is also not a good approach because recombinant PTN produced in baculovirus, yeast, or E. coli does not retain the full-range of activities and the recombinant protein produced in mammalian cells is impure and losses its activity when purified [Fang, 1992 #2530]. 3] Therefore, to test our hypothesis, we decided to clone the full-length human PTN cDNA and generate stably transfected THP-1 and RAW monocytic cells using a bicistronic retrovirus vector.

Cloning of human PTN gene

We used the full-length human PTN open reading frame (accession # NM_002825) to clone the full-length cDNA. The PTN cDNA was generated by reverse transcription of human brain polyadenylated mRNA (Clontech) and amplification via polymerase chain reaction with specific primers for PTN (5'AAAATGCAGGCTCAACAGT AND 5'TGTTTGCTGATGTCCTTT). The PCR product was cloned into pCRII-TOPO vector (Invitrogen) and five clones were selected for further analysis. Nucleotide sequence analysis of the clones revealed that they contain full-length PTN cDNA (3 clones of sense and 2 clones of anti-sense orientations). To further validate the sequence veracity, it was electronically translated into protein using the ExPASY Translate tool program and its molecular weight was determined by the ExPASY Compute pI/Mw tool program. The theoretically translated product was composed of 136 amino acids with a theoretical molecular weight of 15.3 kDa and a pI=10.3. We concluded that the cloned cDNA sequence matched the full-length PTN nucleotide and amino acid sequences found in the GenBank database. After feeling confident about the cloned PTN gene, we subcloned the gene into a retroviral vector in order to transduce monocytic cells.

Generation of retroviral vectors, retrovirus production, and infection of monocytic cells

We constructed a bicistronic retroviral vector for our experiments. The bicistronic retroviral vector was constructed using the pLP-EGFP-C1 plasmid (Clontech) containing the enhanced green fluorescent protein (GFP) gene under the control of the CMV promoter. PTN cDNA was positioned down-stream to the CMV promoter. Next, we cloned an internal ribosomal entry site (IRES) sequence downstream of PTN and upstream of GFP in order to generate the bicistronic retroviral vector (Fig. 2). The IRES in this vector permits simultaneous expression of PTN and GFP from one mRNA. This bicistronic retroviral vector has several advantages over monocistronic vectors: 1] it allows us to follow PTN gene expression in infected cells *in vitro* and *in vivo* by monitoring for GFP expression, 2] PTN translation occurs independent of GFP allowing for the secretion of PTN from cells while GFP remains in the cells, and 3] transduced cells can be isolated by FACS.

Figure 2. Structure of the retroviral expression vector containing the human PTN cDNA. The pLP-GFP-C1 retroviral expression plasmid was modified with the insertion of human PTN cDNA (first cistron) and an IRES sequence between the CMV promoter and the GFP (second cistron). The first gene was positioned either in a sense or anti-sense orientation after the CMV promoter.

All retroviral expression plasmids were constructed using the pLP-C1-IRES-GFP (Clontech) retroviral vector and standard molecular biology techniques. In these cassettes, transcription is initiated by promoter sequences within the viral 5' long terminal repeat (LTR) and terminated by polyadenylation sequences within the 3' LTR. Translation of the first (PTN) and second (GFP) cistrons from a single mRNA proceeds by ribosome binding to 5' Cap and IRES sequences, respectively. The full-length cDNA of human PTN was cloned into the *BamH/Notl* sites of pLP-C1-IRES-GFP. The retrovirus was packaged using a 293 packaging cell line provided by Clontech. After transfection of packaging cells, the medium was changed at 10 h and again at 24 h after transfection. Virus collected between 24 and 48 h after transfection was used for infection. Retroviral titers between $1x10^6$ and $2x10^7$ cfu/ml were determined by limiting dilution with NIH3T3 cells. For infection, $4x10^5$ human THP-1 or mouse RAW monocytic cells were plated in 25-cm^2 flasks 24 h before infection in normal growth medium (DMEM/10%FBS) to obtain exponentially growing cultures. The medium was replaced with 4 ml of retroviral supernatant (approximate MOI 2.5-25 cfu/cell) supplemented with 4 pg/ml polybrene. After 12 h, retroviral supernatants were removed and replaced with fresh normal medium for 48 h. Under these conditions, no apparent toxicity was observed after a 12-h exposure to retroviral supernatants containing polybrene in cultured cells.

Next, we asked whether GFP reporter gene expression could be used to isolate distinct

populations of PTN-expressing cells and if gene expression is maintained over multiple passages in culture. Cultured monocytic cells were infected with PTN-IRES-GFP virus or control IRES-GFP virus. Polyclonal populations of cells ($3x10^5$ each) expressing low or high levels of the GFP reporter gene were then isolated by flow cytometry. Immediate post-sort analysis confirmed the isolation of distinct populations of cells based on GFP expression (not shown). Cells were subcultured for an additional passage and analyzed for expression of the GFP reporter gene by flow cytometry and for expression of PTN by Western blot analysis (see below). Sorted cells maintained their relative expression levels of GFP, and there was excellent correlation between GFP and PTN expression. We concluded that GFP reporter gene expression can be used to isolate distinct populations of cells expressing different levels of PTN by flow cytometry.

As shown previously in Fig. 1, human THP-1 and mouse RAW monocytic cells do not express PTN under basal condition and activation of monocytic cells by TNF-α was required for the expression of PTN. Therefore, we used Northern and Western blot analyses to investigate expression of PTN in the infected monocytic cells. Consistent with data in Fig. 1, we found that unactivated monocytic cells do not express PTN mRNA; however, cells infected with PTN sense and anti-sense strand expressed PTN mRNA (not shown). PTN mRNA was not detected in the cells infected with the GFP control vector. Western blot analysis revealed that PTN gene product is expressed by THP-1 or RAW cells that have been infected with PTN sense strand, but not infected with PTN anti-sense strand or control GFP vector (not shown). After feeling confident about the expression of PTN by monocytic cells, we asked what is the role of PTN in macrophage-mediated angiogenesis.

What is the role of PTN in macrophage-mediated tumor neovascularization?

As discussed above, based on close embryological association between endothelial cells and monocytes and the ability of differentiated cells to switch their fate, we reasoned that monocytes could be converted into endothelial cells. Specifically, we hypothesize that PTN promotes transdifferentiation of infiltrating monocytes into endothelial-like cells thus stimulating tumor neovascularization. A corollary hypothesis would hold that PTN is a plastogenic factor.

To test our hypothesis, we examined the expression of several established endothelial cell markers in PTN-infected cells. The markers were: vascular endothelial growth factor receptor-2 (Flk-1)[Yamaguchi, 1993 #3144], Tie-2[Sato, 1993 #3049], vascular endothelial-cadherin (VE-cad)[Lampugnani, 1992 #3142], PECAM-1[DeLisser, 1994 #3141], endothelial nitric oxide synthase (eNOS)[Moncada, 1991 #3146], the von Willebrand factor (vWf)[Moncada, 1991 #3146], and CD34 [Young, 1995 #3143]. Total cellular RNAs were isolated by using RNEasy Mini Kit (Qiagen, Chatsworth, CA). RT-PCR was performed using Qiagen OneStep RT-PCR kit with the addition of 10 units Rnase inhibitor (GIBCO/BRL) and 40 ng RNA. The primer sequences and PCR conditions are included in the appendix. Primer sequences for each specific marker were designed using human DNA sequences from the GenBank database. To ensure semi-quantitative results of the RT-PCR assays, the number of PCR cycles for each set of primers was checked to be in the linear range of the amplification. In addition, all RNA samples were adjusted to yield equal amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard. The amplified products were separated on 1.2% agarose gels with ethidium bromide (E-Gel, Invitrogen).

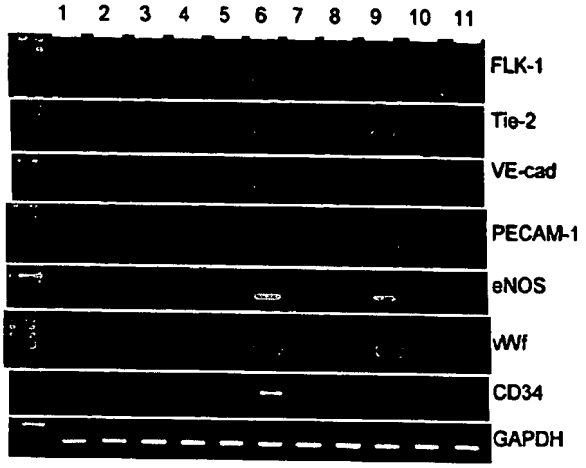


Fig.3. RT-PCR analysis of endothelial cell marker expression. Total RNA was isolated from various cell types and subjected to RT-PCR analysis. The PCR conditions were shown in the appendix. The GAPDH was used as an internal standard.

Infected THP-1 cells and uninfected monocytic cells were analyzed by semi-quantitative PCR for the expression of endothelial cell markers. As a comparison, the expression pattern of these markers was analyzed in non-monocytic NIH 3T3 cells, human smooth muscle cells, and human skin fibroblasts. All cell lines were obtained from ATCC and cultured according to their instructions. The analysis from three independent experiments is shown in Fig. 3. THP-1 cells infected with PTN sense strand readily expressed detectable levels of endothelial cell markers (lane 9), similar to that of positive control human coronary artery endothelial cells (lane 6). In contrast, the expression of these markers was not detectable in uninfected mouse monocytic RAW cells (lane 1), human promonocytic leukemic U937 cells (lane 2), and THP-1 cells (lane 3). Similarly, these markers were not detectable in NIH 3T3 cells (lane 4), human smooth

muscle cells (lane 5), RPMI 8226 B lymphocyte plasmacytoma cell line (lane 7), and human skin fibroblasts (lane 8). In addition, these endothelial cell markers were not detected in either THP-1 cells infected with PTN anti-sense strand (lane 10) or GFP control vector (lane 11). The weak expression of FLK-1 and Tie-2 in smooth muscle cells (lane 5) is consistent with the previous report on the expression of these endothelial cell markers in human smooth muscle cells [Ishida, 2001 #3138]. The expression pattern of these endothelial cell markers in PTN-infected RAW cells was similar to PTN-infected THP-1 cells (not shown).

These data clearly demonstrate that uninfected monocytic and non-monocytic cells do not express endothelial cell markers. However, infection of THP-1 or RAW cells with PTN sense strand markedly up-regulates expression of established endothelial cell markers. In addition, since the number of PCR cycles for each set of primers was chosen to be in the linear range of the amplification, these data show that the expression levels of endothelial cell markers are similar to those of positive control endothelial cells. Further, PTN appears to up-regulate expression of endothelial cell markers that are representative of mature endothelial cells such as VE-cadherin, PECAM-1, eNOS, and VWF, suggesting that PTN-infected THP-1 cells assume a phenotype of mature endothelial-like cells.

Several studies have suggested the existence of multipotent adult stem cells that have the potential to replenish several cell lineages in various tissues, even across the germ layer barrier [Orkin, 2000 #3053]. It is thought that adult hematopoietic stem cells could retain plasticity and therefore, capable of regenerating multiple cell types in nonhematopoietic tissues, including endothelial cells [Jiang, 2002 #3132]. The monocytic cell lines that we have used in our studies are established cell lines with known monocytic cell characteristics; therefore, they do not have multipotent characteristics like adult hematopoietic stem cells. The results of PCR studies described above clearly support this and show that the uninfected cells do not express endothelial cell markers. However, to further examine whether the monocytic cells that we used have characteristics of stem cells or immature endothelial cells, we examined the expression of zinc finger transcription factors known to be expressed in immature and mature endothelial cells such as GATA-2 and GATA-3 [Lee, 1991 #3150;Gumina, 1997 #3151;Jahroudi, 1994 #3152;Cowan, 1998 #3153], as well as the expression of Oct-4 transcription factor known to be expressed in pluripotent cells [Yeom, 1996 #3139].

Semi-quantitative PCR analysis (Fig. 4) showed that THP-1 cells infected with PTN sense strand (lane 9) expressed these transcription factors and that the expression levels of the factors are similar to control human endothelial cells (lane 6). In sharp contrast, uninfected monocytic RAW, U937, and THP-1 cells (lanes 1-3) as well as THP-1 cells infected with either PTN anti-sense strand (lane 10) or GFP control vector (lane 11) did not express the transcription factors. In addition, non-monocytic NIH 3T3 cells (lane 4), smooth muscle cells (lane 5), RPMI 8226 B lymphocyte plasmacytoma cells (lane 7), and human skin fibroblasts (lane 8) also did not express GATA-2 and GATA-3.

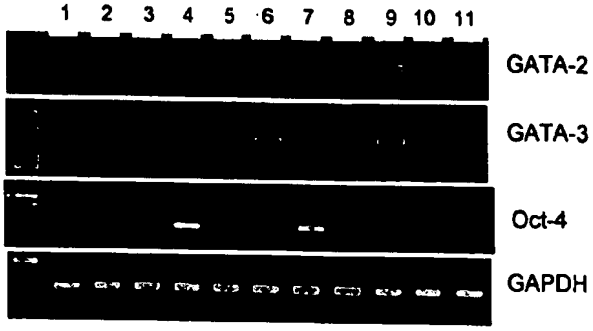


Fig.4. Expression of transcription factors. Total RNA was extracted from each cell type and RT-PCR was performed as described in Fig. 3.

We also used semi-quantitative PCR to investigate the expression of Oct-4 transcription factor. PCR analysis revealed that none of the monocytic cells examined expressed Oct-4, suggesting that they have a mature phenotype. Similarly, control non-monocytic cells such as smooth muscle cells, endothelial cells, and human fibroblasts did not express Oct-4. Interestingly, NIH 3T3 cells (lane 4) and RPMI 8226 B lymphocyte plasmacytoma cell line (lane 7) expressed Oct-4 suggesting that they have characteristics of immature cells.

The results of PCR experiments described above led us to hypothesize that PTN infection of THP-1 and RAW monocytic cells induces transdifferentiation of the cells into endothelial-like cells. We consider this process to be transdifferentiation because it meets the two criteria required for this event [Eguchi, 1993 #3043]: 1) The two types of differentiated states before and after transdifferentiation is clearly defined (monocytic cell lines vs. endothelial cells), 2) the cell types are of two different lineages (monocytic lineage vs. endothelial lineage).

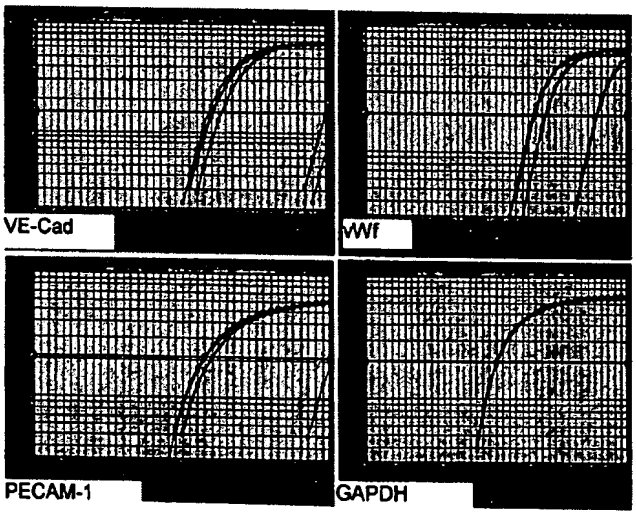


Fig. 5. Real-time PCR analysis of selected endothelial cell markers. GAPDH was used as an internal control.

Although our semi-quantitative RT-PCR

showed that the level of expression of endothelial cell markers in THP-1 and RAW cells infected with PTN sense strand is similar to the positive control endothelial cells, we used an additional approach, real-time PCR (TaqMan), which is more sensitive and quantitative, to confirm the expression levels of selected endothelial cell markers in infected THP-1 cells. Using primers specific for VE-cadherin, vWf, and PECAM-1, we found that the expression levels of these endothelial cell markers in THP-1 cells infected with PTN sense strand (Fig. 5, blue line) are similar to that of positive control endothelial cells (red line), but not in cells infected with the GFP control vector (yellow line). Similar results were obtained with Tie-2, and VE-cadherin (not shown). GAPDH amplification was used as an internal standard. These results confirm the semi-quantitative RT-PCR data and clearly show that the expression level of endothelial cell markers in PTN sense strand-infected THP-1 cells is similar to endothelial cells, suggesting that they may be biologically relevant.

We also used an immunohistochemical panel of endothelial cell markers (FLK-1, Tie-2, VE-Cadherin, PECAM-1) to determine the spatial distribution of the markers. The antibodies were obtained from Santa Cruz, Inc. and used at the recommended dilutions. Immunostaining was performed as we described previously [Wallner, 1999 #2319]. Immunohistochemical analysis of THP-1 cells infected with PTN sense strand showed strong expression of FLK-1 marker on the surface of cells (Fig. 6, right panel). No expression of FLK-1 was detected in THP-1 cells transduced with GFP control vector (left panel). Similar results were obtained with Tie-2, VE-Cadherin, and PECAM-1 (not shown).

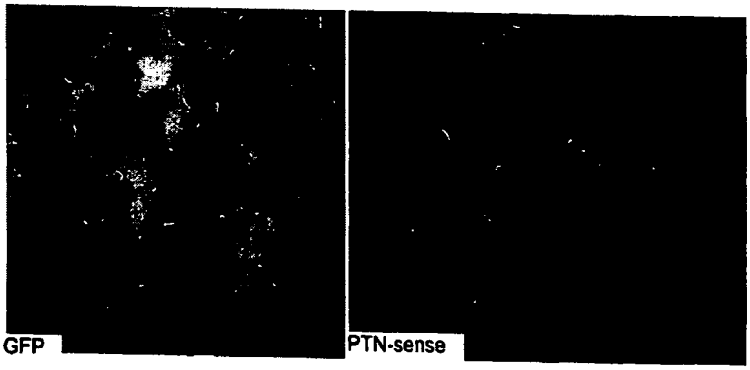


Fig. 6. Immunostaining analysis of PTN-infected THP-1 cells. Cultured THP-1 cells were fixed and then treated with by anti-FLK-1 antibody followed by secondary antibody. 20X magnification

To precisely determine the topographical relationship between distribution of transcription factors and endothelial cell markers in THP-1 cells infected with PTN sense strand, we performed double staining of the infected cells with anti-human GATA-2 rabbit polyclonal antibody (obtained from Santa Cruz) and anti-human VE-cadherin mouse monoclonal antibodies. A representative confocal image of THP-1 cells infected with PTN sense strand is

shown in Fig. 7. THP-1 cells infected with PTN sense strand and GFP control were cultured on coverslips and stained with anti-VE-cadherin monoclonal antibody. Afterwards, cells were permeablilized followed by staining with anti-GATA-2 rabbit polyclonal antibody (obtained from Santa Cruz). The secondary antibodies (Alexa Fluor 633 goat anti-rabbit antibody and Alexa Fluor 568 goat anti-mouse antibody) were used at 1:500 dilutions, as recommended by

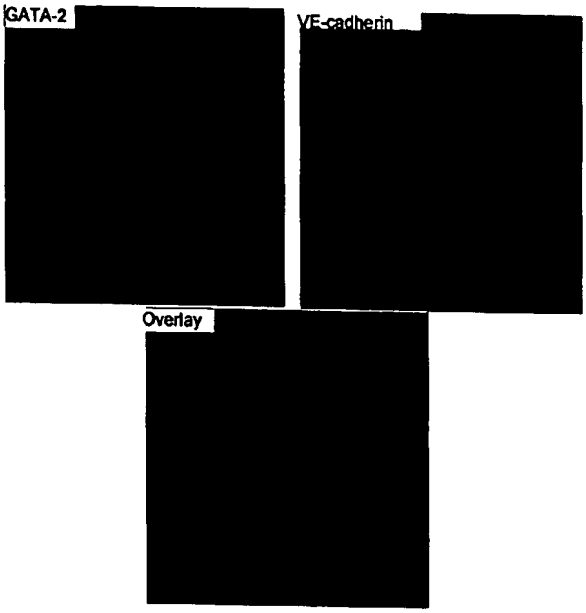
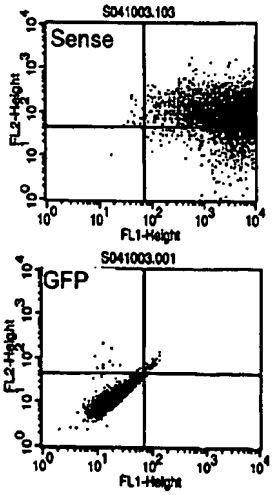


Fig. 7. Representative confocal image of THP-1 cells infected with PTN sense strand. The infected cells were permelized with 0.2% Tween 20 followed by staining with anti-VE-cadherin monoclonal antibody or anti-GATA-2 polyclonal antibody. The red color represents VE-cadherin and blue color shows GATA-2.

Molecular Probe. Consistent with the light microscopy results, we found strong expression of VE-cadherin on the surface of PTN-infected cells (red color). As expected, GATA-2 expression was concentrated in the nucleus (blue color). The overlay clearly showed the co-expression of two endothelial cell markers in THP-1 cells infected with PTN sense strand. No VE-cadherin or GATA-2 staining were detected in THP-1 cells infected with the control vector (not shown).

We also examined the expression of another endothelial cell marker, $\alpha_v\beta_3$ integrin, in infected THP-1 cells. We selected this integrin because the interaction between $\alpha_v\beta_3$ integrin and extracellular matrix is crucial for endothelial cells sprouting from capillaries and for angiogenesis [Soldi, 1999 #3181]. In addition, $\alpha_v\beta_3$ integrin participates in the full activation of FLK-1 which is known to be important in tumor angiogenesis, inflammation, and tissue regeneration [Soldi, 1999 #3181]. Using a 1:100 dilution of $\alpha_v\beta_3$ antibody (obtained from Chemicon Co) and FACS analysis, we found that 82% of positive control human



endothelial cells expressed $\alpha_v\beta_3$ integrin (not shown). Similarly, 88% of THP-1 cells infected with PTN sense strand expressed $\alpha_v\beta_3$ integrin (Fig. 8, right panel, Sense). In contrast, less than 1% of THP-1 cells infected with GFP control retrovirus expressed $\alpha_v\beta_3$ integrin (Fig. 8, right panel, GFP). We conclude that PTN induces signaling events in THP-1 cells that are important for endothelial cell activity and survival.

As a final *in vitro* study, we investigated the ability of the transduced THP-1 and RAW cells to form tubular structures. PTN- and GFP-transduced cells were cultured on three-dimensional fibrin matrices in RPMI/10% serum media. The fibrin gel was prepared essentially as described [Koolwijk, 1996 #3212]. Uninfected THP-1 and RAW cells were used as negative controls and human endothelial cells were used as a positive control. After 3 days in culture, cells infected with PTN sense strand invaded the fibrin matrix and started to form network-like structures in the three-dimensional gel, similar to positive control endothelial cells (not shown). In contrast, uninfected THP-1 and RAW cells as well as cells infected with the control GFP vector remained on top of the fibrin matrix and no network-like structure could be observed (not shown). These data demonstrate that infection of monocytic cells with PTN confers ability to the cells to rearrange in the fibrin gel with extended cytoplasm and interact with surrounding cells, similar to endothelial cells.

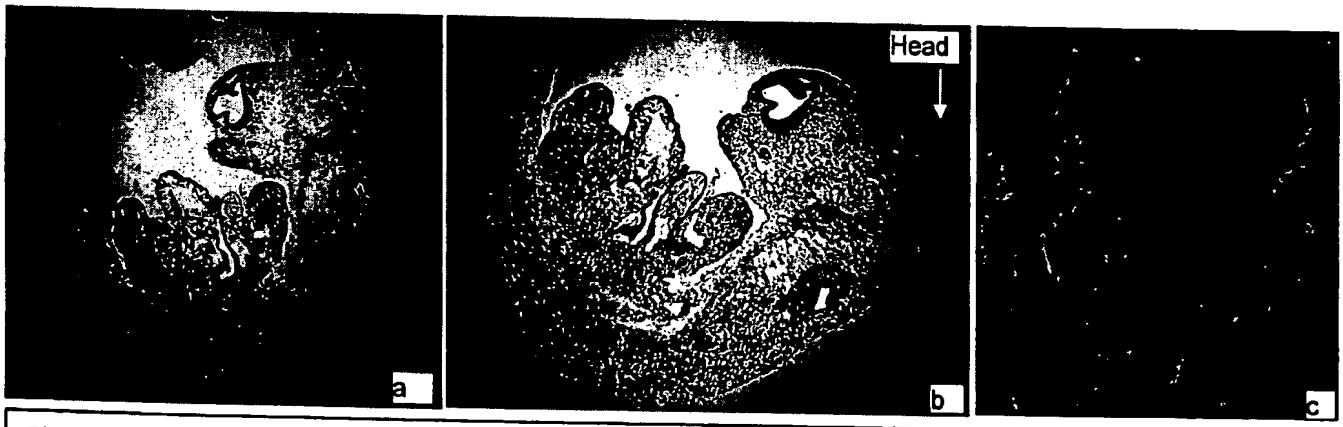


Fig. 9. Contribution of RAW cells stably transduced with PTN to vascular formation *in vivo*. Frozen sections of chick embryos 3 days after injection with RAW cells expressing only GFP (panel a) or PTN sense strand plus GFP (b). The sections were stained with 1:100 dilution of anti-GFP antibody (Santa Cruz). Panel C shows high resolution confocal image of chicken embryo head.

Based on the outcome of the *in vitro* experiments, we decided to study the potential of the PTN-infected cells to incorporate into the newly formed vasculature using a chick/mouse

chimeric assay. We injected 1-2 x10^5 cells in 2-4 µl into the hearts of stage 16-17 chick embryos with glass needles. Embryos were killed 2-3 days after injection, fixed, embedded in OTC, and frozen sections were cut and stained with anti-GFP antibodies. As shown in Fig. 9, the chicken embryo injected with RAW cells expressing only GFP did not stain with GFP antibody (panel a). In contrast, injection of RAW cells expressing both PTN and GFP (panel b) showed GFP staining (brown color) concentrated along blood vessels in the head, eyes, heart, and intersomitic region, and in some cases, forming a network structure 2-3 days after injection. Confocal image of the chicken embryo from panel b showed the association of GFP signal with microvessels in the head region (panel c). Tie-2 antibody staining pattern showed a similar pattern of staining (not shown).

In summary, our data clearly show that the uninfected monocytic cell lines that we have used do not have endothelial cell characteristics. The cells acquired endothelial cell markers when cells are transduced with retrovirus harboring PTN sense strand, but not infected with either PTN anti-sense strand or control GFP vector. Immunostaining data showed that the endothelial cell markers normally found on the surface of endothelial cells such as Tie-2, FLK-1, and VE-cadherin are expressed on the cell membrane of PTN-transduced cells indicating that the markers have correct topological configuration. The monocytic cells infected with PTN sense strand express $\alpha_v\beta_3$ integrin, suggesting that they have the potential to interact with extracellular matrix components that are found in the vasculature. The infected cells are also capable of forming tubular structure *in vitro* and found to be incorporated into newly formed vessel of developing chicken embryo, suggesting that they behave like endothelial cells both *in vitro* and *in vivo*. Overall, we offered evidence that PTN coaxed monocyte/macrophage lineage into endothelial-like lineage, suggesting that there may be merit in studying this event in more detail.

We would like to emphasize that our finding of endothelial cell generation from monocytic cells is clearly different from the other existing models of endothelial cell formation. Our finding is clearly different from an angiogenesis model where endothelial cells are derived from pre-existing vasculature. In addition, our finding is clearly different from a vasculogenesis model where endothelial cells originates either from maturation of hemeangioblasts or angioblasts. The THP-1 and RAW cell lines that we used are widely known differentiated monocytic cells that (i) do not express endothelial cell markers or (ii) express markers indicating that they do not have an endothelial progenitor cell phenotype. In addition, our finding is

different from the vascular mimicry model proposed by Maniotis *et al.* [Maniotis, 1999 #3168] where vascular channels are formed without the participation of endothelial cells and independent of angiogenesis. According to the vascular mimicry model, tumor cells themselves either carry blood or connect to the host's blood supply [Folberg, 2000 #3169]. These channel-forming tumor cells do not express endothelial cell markers [Weidner, 2002 #3223]. Our finding is different from the vascular mimicry model because the transdifferentiated monocytic cells express endothelial cell markers, unlike the cells that form blood channels in the model. Despite differences, there are some similarities between our finding and vasculogenesis or vascular mimicry models. Our finding shares some resemblance to the vasculogenesis model of blood vessel formation owing to the *de novo* nature of endothelial cell generation. Our finding also shares some similarity to the vascular mimicry model owing to the formation of endothelial cells without participation of either endothelial cells or its progenitor cells.

We concentrated on PTN as a potential plastogenic factor in part, because of recent finding described by Sugino *et al.* [Sugino, 2002 #3077]. They compared gene expression profiles of highly invasive metastatic murine mammary tumor cells with those of non-metastatic cells and found that pleiotrophin (PTN) is selectively up-regulated in the invasive metastatic cells. Although the expression of endothelial cell markers in these metastatic cells were not investigated, PTN expression was found to correlate with the ability of the metastatic cells to form vasculature and connect to blood vessels without participation of pre-existing blood vessel endothelial cells. However, we do not know whether this plastogenic activity is specific to PTN or other angiogenic factors have similar properties. In addition, we do not know whether other hematopoietic cells or tumor cells have the potential to transdifferentiate into endothelial-like cells by PTN or that the effect is restricted to monocytic cells. Regardless of these issues, the finding that the endothelial cells required for blood vessel formation need not originate from either pre-existing endothelial cells or their precursors, but rather are generated *de novo* from other cell types provides a new target to control tumor growth and metastasis.

Research Design and Methods

Aim 1) Does PTN induces transdifferentiation of glioma cells?

To test our hypothesis that cell transdifferentiation into endothelial cells contributes to tumor vascularization, we will focus on glioma tumors for two reasons. 1] Gliomas are highly

vascularized tumors and an efficient blood supply is of critical importance for their growth. 2] Recently, PTN was found to be expressed by human glioma cell lines, cell cultures derived from solid gliomas, and glioma sections [Mentlein, 2002 #3219]. PTN mRNA or protein was detected in all WHO III and IV grade gliomas and cells analyzed *in vitro* or in situ. In situ, PTN expression was restricted to distinct parts/cells of the tumor. PTN showed a strong chemotactic effect on murine BV-2 microglial cells [Mentlein, 2002 #3219]. The human *PTN* gene is localized on chromosome 7, band q33-34 [Li, 1992 #3022]. This chromosome is often amplified in gliomas [Sehgal, 1998 #3221], and therefore enhanced PTN expression may result from this malignant transformation.

This aim will expand our preliminary data by asking whether the transdifferentiation activity of PTN is restricted to monocytic cells or PTN can also induce glioma cells to transdifferentiate into endothelial-like cells. We hypothesized that PTN is a plastogenic factor that promotes tumor neovascularization through transdifferentiation of cells into endothelial-like cells. To test this idea, we will express PTN in glioma cells and determine the phenotypic characteristics of the infected cells.

We have already made the retrovirus expressing PTN and the preliminary data show that the retrovirus infects macrophage cells and the infected cells express PTN. We will use an equivalent approach to infect glioma cells. To save space, we will not repeat the methodology to infect glioma cells. We have already received two glioma cell lines U 87 and MO59 that are growing in our laboratory. The exponentially growing cells will be infected with the bicistronic retrovirus harboring GFP, or GFP+PTN sense strand or GFP+PTN anti-sense strand. The infected cells will be separated from uninfected cells by using G418 selection media and FACS. The expression of PTN in the infected cells will be assessed by Northern and Western blot analyses using the probes that we have developed. The expression of endothelial cell markers will be evaluated by RT-PCR and immunostaining as described above. In addition, we will determine the expression of GATA-2, GATA-3, and Oct-4 transcription factors in the uninfected and infected cells.

Outcome:

We do not anticipate any major technical hurdles in carrying out the experiments. We have the expertise and the reagents necessary to perform the experiments.

Previous studies have shown that glioma cells express PTN. If, PTN converts glioma

cells into endothelial cells, we anticipate that the uninfected glioma cells will express endothelial cell markers. To our knowledge, there has been any report about the expression of endothelial cell markers in glioma cells.

It is possible that we do not detect expression of endothelial cell markers in uninfected cells, whereas glioma cells infected with PTN sense strand express endothelial cell markers. One explanation for such a finding would be gene dose effect. We do not know the level of PTN gene expression that is required for the conversion of monocytic cells into macrophages. In addition, we do not know whether the level of endogenous PTN produced by glioma cells would be sufficient for conversion of cells into endothelial cells. In addition to tumor cells, macrophages are a major component of the leukocyte infiltrate of tumors [Balkwill, 2001 #3258]. Tumor-associated macrophages have complex dual functions in their interaction with neoplastic cells, and evidence suggests that they are part of inflammatory circuits that promote tumor progression [Balkwill, 2001 #3258]. Our preliminary data showed that activated macrophages express PTN. Thus, in tumors, there appear to be two sources of PTN: tumor cells and macrophages. It is possible that the level of endogenous PTN produced by tumor cells *per se* may not be sufficient for the conversion of tumor cells into endothelial cells. Additional source of PTN, such as macrophages, may be required to generate enough of a dose of PTN necessary for transdifferentiation to occur. Therefore, the uninfected glioma cells may not express endothelial cell markers whereas cells infected with PTN sense strand express sufficient dose of PTN required for transdifferentiation processes to occur.

Previously, Choudhuri *et al.* [Choudhuri, 1997 #3193] overexpressed PTN in MCF-7 cells, a breast carcinoma cell line. Although the authors did not investigate the phenotypic modulation of cells by PTN, they did showed that PTN-infected MCF-7 cells produced tumors that grew significantly faster than uninfected cells or cells transfected with a control DNA plasmid. Furthermore, these PTN-induced tumors had a greater vascular density compared to control tumors. In a corneal angiogenesis assay, it was shown that corneas receiving PTN-infected MCF-7 cells scored a higher angiogenic response when compared to using uninfected cells. It was found that the vascular pattern in the PTN-expressing tumors was strikingly different from those expressing VEGF. While MCF-7 cells expressing VEGF elicited an angiogenic response within 48 h [Zhang, 1995 #3196], those expressing PTN required 2 weeks [Choudhuri, 1997 #3193]. We suggest that this relatively long incubation time required to

induce angiogenesis by PTN-infected MCF-7 cells may be related to a phenotypic alteration of MCF-7 cells into endothelial-like cells. This aim will explore this possibility. We anticipate that we will observe similar activity for glioma cells infected with PTN compared to uninfected glioma cells.

Aim 2) What is the active domain of PTN?

This aim will extend the preliminary data by asking which segment or domain of the PTN molecule is responsible for its transdifferentiation activity. We hypothesize that the potential of PTN to induce transdifferentiation is mediated by several functional domains.

Prior studies have demonstrated that PTN is a mitogen and utilized a series of constructed PTN mutant proteins to determine the domains required for the transformation activity [Zhang, 1999 #3187]. It was found that a combination of two PTN segments was required for the transformation activity. However Inui *et al* [Inui, 2000 #3194] showed that only one segment corresponding to the N-terminal domain of PTN was required for transformation activity of PTN. Others have shown that while the C-terminal end of PTN was required for transformation activity, the N-terminal segment retained its neurite outgrowth activity [Bernard-Pierrot, 2001 #3195], suggesting that mitogenic and neurite outgrowth activities are mediated through different pathways. Employing a similar strategy, we will construct a series of PTN mutants and test them for their ability to transdifferentiate THP-1 and RAW cells.

The PTN mutants will be constructed with consideration to (i) the N- and C-terminals which contain heparin-binding β-sheet domains [Kilpelainen, 2000 #3189] and (ii) the middle portion that is a flexible linker between the terminal ends and is associated with transformation activity [Zhang, 1999 #3187]. We have already cloned full-length human PTN. We will design PCR primers corresponding to the N-and C-terminal halves of the protein. Using the full-length PTN as a template, we will amplify the N- and C-terminal domains of PTN and the veracity of nucleotide sequence will be verified by DNA sequencing. The PCR products will be cloned into the TOPO PCR-2 vector (Invitrogen), followed by subcloning into a retroviral bicistronic vector that we have developed (discussed above), using standard molecular biology techniques. As described above, the retroviral vectors will be packaged, viral titers will be determined, and monocytic cells will be infected with the viruses. The expression of endothelial cell markers will be assessed as discussed above using PCR and immunohistochemical staining. Endothelial cell

markers (e.g., FLK-1, Tie-2, VE-cadherin, PECAM-1, endothelial nitric oxide synthase and the von Willebrand factor) will be utilized to determine the extent of transdifferentiation *in vitro*. The ability of infected cells to promote tumor vascularization *in vivo* will be assessed by standard xenograft transplantation experiments using PTN-infected glioma cells or human THP-1 monocytic cells. Infected cells will be injected subcutaneously at a unique site in mice. Tumor size will be measured twice a week, starting from the second week following injection. Mice will be sacrificed 6 weeks after injection.

Outcome:

We do not anticipate major technical problems in carrying out the experiments described above. We have already cloned full-length wild type PTN and the retrovirus that we have generated is functional and capable of infecting cells. The deletion experiments are straightforward.

Previous study has shown that infection of breast cancer cells with a truncated mutant of PTN reverted the transformed phenotype of the breast cancer cells [Zhang, 1997 #3179]. PTN is a heparin binding protein and its N- and C-terminal domains have a strong net positive charge, suggesting they could interact with the receptor or an associated second "low affinity" receptor through electrostatic forces but are unlikely to signal active site-mediated receptor functions. Recently, N-syndecan has been implicated as a PTN binding protein [Raulo, 1994 #3031], however, the binding of N-syndecan to PTN is not specific to PTN since basic fibroblast growth factor (bFGF) competes for PTN binding sites, and the glycosaminoglycan chains alone of N-syndecan bind both PTN and bFGF [Raulo, 1994 #3031]. N-syndecan functions as a low affinity receptor and appears to regulate binding of bFGF to its high affinity receptor [Chernousov, 1993 #3262]. There is no data about the specific receptor that mediates interaction of PTN with either macrophages or glioma cells. In the complete absence of information about the receptor, it is difficult to predict the outcome of experiment. However, our data will provide a structural basis for further studies on the functions of PTN in transdifferentiation in glioma cells and other human tumors. Our findings also will provide a molecular model system to dissect the functional responses in tumors constitutively expressing PTN.

Aim 3) What is the downstream signaling in PTN-mediated transdifferentiation?

This aim will extend the effort of aim 2 by investigating the PTN-induced signaling in

monocytes/macrophages. Currently, nothing is known about PTN signaling in monocytes/macrophages. As an initial step to understand PTN signaling involved in transdifferentiation of monocytes/macrophages to endothelial cells, we will concentrate our effort on the mitogen-activate protein (MAP) kinase pathway for two reasons: 1] this pathway is known to be activated by PTN in bovine epithelial lens cells [Souttou, 1997 #3086], and 2] MAP kinase pathway is thought to be a key signaling pathway that have been implicated in the phenotypic outcome for endothelial cells and angiogenesis [Lee, 2000 #3213]. Activation of MAP kinase pathway has been investigated in a variety of macrophages including THP-1 [McGilvray, 1998 #3198;Hambleton, 1996 #3199;Huang, 1999 #3200;Kurosawa, 2000 #3201;Bowling, 1996 #3202] and RAW [Hambleton, 1996 #3203;Chen, 1999 #3204;Ajizian, 1999 #3205;Petrache, 1999 #3206] cells. We will use inhibitors of MAP kinase to block the transdifferentiation of THP-1 and RAW cells transduced with PTN retroviruses. Control cells will include uninfected cells and cells infected with retroviruses carrying only the GFP reporter gene. After infection with the retroviruses, cells will be allowed to establish an efficient level of transduction for 24-48 hours. Cells will then be treated with inhibitors of MAP kinase which known to be effective in THP-1 and RAW cells such as SB203580 [Lee, 1994 #3208] (Calbiochem, San Diego, CA). After treating for various time points, the expression of endothelial cell markers will be assessed, as we described above. To confirm the activation of the MAP kinase signaling pathway in cells undergoing transdifferentiation, we will perform established Western blot analysis to assess phosphorylation of specific protein substrate targets (e.g. SHC, ERK1, ERK2, and Akt1) in the PTN-transduced cells. Control cells that are not undergoing transdifferentiation should display lower levels of these phosphorylated proteins.

Outcome:

We predict that the MAP kinase inhibitors will block the transdifferentiation activity of PTN, suggesting that this pathway is involved in PTN-mediated transdifferentiation of monocytes/macrophages into endothelial-like cells. Alternatively, it is possible that the transduced THP-1 and RAW cells have already committed to the endothelial cell phenotype during the initial period of retroviral transduction and therefore, the inhibitors remain ineffective. To address this issue, we will add the inhibitors just prior to retrovirus infection in an attempt to block the transdifferentiation from proceeding. It is also possible that other pathways are responsible for PTN-induced signaling in monocyte/macrophages. As an initial step to explore

such possibility, we will focus on the activation of Src which as been postulated as an intermediary signaling molecule for PTN activity [Muramatsu, 2002 #3021].

F. VERTEBRATE ANIMALS

Proposed animal use. We will use standard xenograft transplantation experiments for *in vivo* studies. Infected cells will be injected subcutaneously at a unique site in nude mice (Jackson Laboratories). Tumor size will be measured twice a week, starting from the second week following injection. Mice will be sacrificed 6 weeks after injection.

Justification for animal use. Xenografts of cancer cells into nude mice have been widely used to investigate tumor vascularization *in vivo*.

Veterinary care. All animals will be maintained at the Burns and Allen Research Institute of the Cedars-Sinai Medical Center. The vivarium at this institution is a registered research facility with the United States Department of Agriculture and is fully accredited by the American Association of Accreditation of Laboratory Animal Care (AAALAC).

Procedures for minimizing pain, discomfort and stress. For transplantation experiment, mice will be anesthetized intraperitoneally with injection of ketamine and xylazine. Post-survival surgery, mice will be given buprenorphine 0.05-0.1 mg/kg SQ.BID (q 12 hr) for a minimum of two doses for analgesia. For euthanasia, mice will be euthanized either by (i) carbon dioxide inhalation followed by decapitation or (ii) decapitation while under anesthesia (ketamine and xylazine).

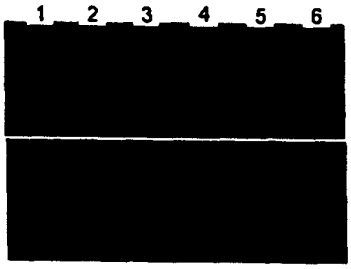
Euthanasia methodology. Animals will be terminated consistent with recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

EXAMPLE 3

Background: Pleiotrophin (PTN), an oncogene produced by breast cancer cells, stimulates cell proliferation in vitro and in vivo. PTN gene is not detected in normal breast cells (1, 2), whereas high levels of PTN expression are detected in 60% of tumor samples from breast cancer patients (3). Cells transformed by the PTN gene form highly angiogenic tumors when implanted into nude mice, whereas a dominant negative PTN mutant inhibits human breast cancer growth in vivo (4). Using expression profiling of non-metastatic and highly invasive metastatic breast cancer cells, Sugino et al (5) recently reported that the PTN gene is differentially overexpressed in metastatic breast cells. The authors implicate PTN as a candidate gene responsible for the neovascularization of metastatic breast cancer cells. It is thought that PTN promotes breast tumor angiogenesis by stimulating endothelial cell recruitment and proliferation (1, 3, 6, 7).

Among breast cancer patients, high vascular grade tumors predict poor survival. High vascular grade also correlates with increased macrophage index (8), and there is a strong relationship between increased macrophage counts and reduced overall survival (8). Tumor associated macrophages are a major source of TNF-α in invasive breast carcinomas (9). It is thought that TNF-α produced by breast tumor associated macrophages regulates expression of angiogenic factors produced by either macrophages or tumor stroma (9). The role of TNF-α in the regulation of PTN expression in macrophages has not been examined and consequently is completely unknown.

Hypothesis: We hypothesized that the expression of PTN in macrophages is regulated by TNF-α. To test this hypothesis, we treated exponentially growing cultured human THP-1 and mouse RAW monocytic cells with TNF-α for 6 h. Control cells were maintained in RPMI/10% bovine serum. In addition, we used exponentially growing human smooth muscle cells (DMEM/10% serum) for comparison. RNA isolation and Northern blot was performed essentially as described (10). Briefly, cultured cells were lysed with guanidine thiocyanate and RNA was isolated by centrifugation through a cesium chloride cushion. The RNA was quantified and 10 μg/lane was analyzed by electrophoresis followed by blotting. The blot was probed with the PTN-specific cDNA probe (top panel). To control for loading, the blot was reprobed with β-actin (lower panel).



Northern blot analysis revealed that untreated, exponentially growing cultured human THP-1 (right panel, lane 1) and mouse RAW (lane 4) cells do not express PTN mRNA. Addition of 10 ng/ml of TNF-α markedly up-regulated expression of PTN in THP-1 cells (lane 2), and in RAW cells (lane 3). Exponentially growing cultured human smooth muscle cells did not express PTN (lane 5) and addition of TNF-α had no effect (lane 6). These data suggest that: 1] the activity of TNF-α in macrophages may in part be mediated by PTN, 2] induction of PTN gene by TNF-α is cell-type specific, and 3] the signaling events activated by TNF-α in the expression of PTN is conserved in human and mouse.

In addition to the effects on neighboring cells, we hypothesized that secreted PTN may affect activity of monocytes/macrophages in an autocrine fashion. There are several options to test this hypothesis: 1] add TNF-α to cells and examine the effect. This would not be a good idea because TNF-α is a pleiotrophic factor that has many known and unknown effects on macrophages which would complicate interpretation of the data. 2] Add recombinant PTN to monocytic cells and evaluate the outcome. This is also not a good approach because recombinant PTN produced in baculovirus, yeast, or E. coli does not retain the full-range of activities and the recombinant protein produced in mammalian cells is impure and losses its activity when purified (1). Therefore, to test our hypothesis, we generated stably transfected THP-1 and RAW monocytic cells using a bicistronic retrovirus vector. We cloned full-length human PTN (accession # NM_002825) using standard molecular biology methods and then subcloned PTN into the bicistronic retroviral vector. The bicistronic retroviral vector was constructed using the pLP-EGFP-C1 plasmid (Clontech) containing the enhanced green fluorescent protein (GFP) gene under the control of the CMV promoter. PTN cDNA was positioned down-stream to the CMV promoter. Next, we cloned an internal ribosomal entry site (IRES) sequence down-stream of PTN and upstream of GFP in order to generate the bicistronic CMV-PTN-IRES-GFP retroviral vector. The IRES in this vector permits simultaneous expression of PTN and GFP from one mRNA. This bicistronic retroviral vector has several advantages over monocistronic vectors: 1] it allows us to follow PTN gene expression in infected cells in vitro and in vivo by monitoring for GFP expression, 2] PTN translation occurs independent of GFP allowing for the secretion of PTN from cells while GFP remains in the cells, and 3] transduced cells can be isolated by FACS.

Next, the retroviral vector was packaged into viral particles using a 293 packaging cell line obtained from Clontech. Virus collected between 24 and 48 h after transfection was used for infection. Retroviral titers between $1x10^6$ and $2x10^7$ cfu/ml were determined by limiting dilution with NIH3T3 cells. For transduction, $4x10^5$ THP-1 or RAW cells were infected with retroviral particles (approximate MOI 2.5-25 cfu/cell) supplemented with 4 pg/ml polybrene. The infected cells were selected by addition of G418 followed by FACS. The integration of PTN gene into THP-1 and RAW cell chromosomes and copy number were confirmed by Southern blot (not shown). The expression of PTN mRNA in THP-1 and RAW cells was validated by Northern blot (not shown). PTN protein expression was verified by Western blot (not shown).

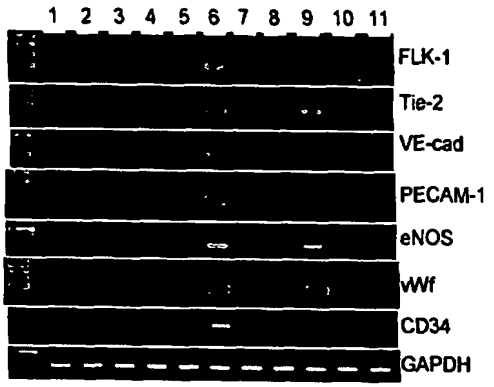
What is the role of PTN in macrophage-mediated tumor neovascularization?

It is known that, in the developing embryo, endothelial cells arise either from progenitor or stem cells that can produce only endothelial cells (angioblasts), or from progenitors that give rise to both endothelial and blood cells (haemangioblasts) (reviewed in (11)). Most of these endothelial precursors divide and differentiate in response to VEGF, which is produced close to where blood vessels are formed (12, 13).

Recent evidence suggests that the ability of immature cells to differentiate into different cell types is not exclusive to progenitor cells. Rather, postmitotic differentiated cells or differentiation-committed cells may also undergo transdifferentiation. For example, hepatic oval cells could be re-differentiated to hormone-producing pancreatic islet cells by culturing in high-glucose medium (14). Conversely, exposure of a pancreatic cell line to glucocorticoid resulted in their transdifferentiation to hepatic cells (15). In a co-culture model, neurosphere-derived cells can differentiate into skeletal muscle cells in the presence of differentiating myoblasts (16). Finally, injection of 3T3-L1 preadipocyte cells into the peritoneal cavity of nude mice converted the cells into macrophages (17). These studies suggest that differentiation is an ongoing process that can be modified by key regulators. Factors that regulate a cell's plasticity are unknown.

Since both endothelial and hematopoietic cell lineages share a common precursor and committed cells are capable of switching their fate, we reasoned that monocytes could be converted into endothelial cells. Specifically, we hypothesize that PTN promotes transdifferentiation of infiltrating monocytes into endothelial-like cells thus stimulating tumor neovascularization. A corollary hypothesis would hold that PTN is a plastogenic factor.

To test our hypothesis, we examined the expression of several established endothelial cell markers in PTN-infected cells. The markers were: vascular endothelial growth factor receptor-2 (Flk-1)(18)), Tie-2(19), vascular endothelial-cadherin (VE-cad)(20), PECAM-1(21), endothelial nitric oxide synthase (eNOS)(22), the von Willebrand factor (vWf)(22), and CD34 (23). Semi-quantitative RT-PCR was performed by using Qiagen OneStep RT-PCR kit with the addition of 10 units RNAase inhibitor (GIBCO/BRL) and 40 ng total RNA isolated from cells by using RNEasy Mini Kit (Qiagen, Chatsworth, CA). Primer sequences for each specific marker were designed using human sequences from the GenBank database. To ensure semi-quantitative results of the RT-PCR assays, the number of PCR cycles for each set of primers was selected to be in the linear range of the amplification. In addition, all RNA samples were adjusted to yield equal amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard. The amplified products were separated on 1.2% agarose gels with ethidium bromide (E-Gel, Invitrogen).

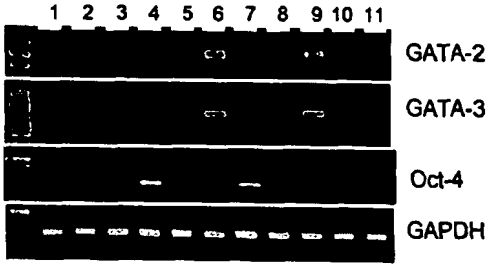


Infected THP-1 cells and uninfected monocytic cells were analyzed by semi-quantitative PCR for the expression of endothelial cell markers. As a comparison, the expression pattern of these markers was analyzed in non-monocytic 3T3 cells, human smooth muscle cells, and human skin fibroblasts. All cell lines were obtained from ATCC and cultured according to their instructions. The analysis from 3 independent experiments are shown in the right figure. THP-1 cells infected with PTN sense strand expressed readily detectable levels of endothelial cell markers (lane 9), similar to that of positive control human coronary artery endothelial cells (lane 6). In contrast, the expression of these markers was not detectable in uninfected mouse monocytic RAW cells (lane 1), human promonocytic leukemic U937 cells (lane 2), and THP-1 cells (lane 3). Similarly, these markers were not detectable in NIH 3T3 cells (lane 4), human smooth muscle cells (lane 5), RPMI 8226 B lymphocyte plasmacytoma cell line (lane 7), and human skin fibroblasts (lane 8). In addition, these endothelial cell markers were not detected in either THP-1 cells infected with PTN anti-sense strand (lane 10) or GFP control vector (lane 11). The weak expression of FLK-1 and Tie-2 in smooth muscle cells (lane 5) is consistent with the previous report on the expression of these endothelial cell markers in human smooth muscle cells

(24). The expression pattern of these endothelial cell markers in PTN-infected RAW cells was similar to PTN-infected THP-1 cells (not shown).

These data clearly demonstrate that uninfected monocytic and non-monocytic cells do not express endothelial markers. However, infection of THP-1 or RAW cells with PTN sense strand markedly up-regulates expression of established endothelial cell markers. In addition, since the number of PCR cycles for each set of primers was chosen to be in the linear range of the amplification, these data show that the expression levels of endothelial cell markers are similar to those of positive control endothelial cells. Further, PTN appears to up-regulate expression of endothelial cell markers that are representative of mature endothelial cells such as VE-cadherin, PECAM-1, eNOS, and VWF, suggesting that PTN-infected THP-1 cells assume a phenotype of mature endothelial-like cells.

We hypothesized that the expression of the endothelial markers shown above should be associated with the expression of transcription factors that are specific to endothelial cells and known to regulate the expression of endothelial cells markers. To test this hypothesis, we examined the expression of zinc finger transcription factors known to be expressed in mature endothelial cells (GATA-2 and GATA-3) (25-28). Semi-quantitative PCR analysis showed that THP-1 cells infected with PTN sense strand (lane 9) expressed these transcription factors and that the expression levels of the factors are similar to control human endothelial cells (lane 6). In sharp contrast, uninfected monocytic RAW, U937, and THP-1 cells (lanes 1-3) as well as THP-1 cells infected with either PTN anti-sense strand (lane 10) or GFP control vector (lane 11) did not express the transcription factors. In addition, non-monocytic 3T3 cells (lane 4), smooth muscle cells (lane 5), RPMI 8226 B lymphocyte plasmacytoma cells (lane 7), and human skin fibroblasts (lane 8) also did not express GATA-2 and GATA-3.



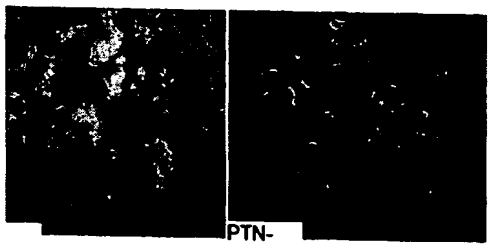
Since immature cells are known to switch their phenotype, we asked whether the THP-1 and RAW monocytic cells that we used have characteristics of progenitor cells or a mature cell phenotype. To distinguish between these two possibilities, we investigated the expression of Oct-4 transcription factor known to be expressed in pluripotent cells (29). Semi-quantitative PCR analysis revealed that none of the monocytic cells examined expressed Oct-4, suggesting

that they have a mature phenotype. Similarly, control non-monocytic cells such as smooth muscle cells, endothelial cells, and human fibroblasts did not express Oct-4. Interestingly, 3T3 cells (lane 4) and RPMI 8226 B lymphocyte plasmacytoma cell line (lane 7) expressed Oct-4 suggesting that they have characteristics of immature cells.

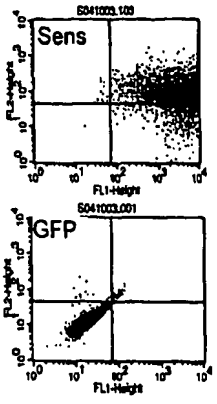
The results of PCR experiments described above led us to hypothesize that PTN infection of THP-1 and RAW monocytic cells induces transdifferentiation of the cells into endothelial-like cells. We consider this process to be transdifferentiation because it meets the two criteria required for this event (30): 1) The two types of differentiated states before and after transdifferentiation is clearly defined (monocytic cell lines vs. endothelial cells), 2) the cell types are of two different lineages (monocytic lineage vs. endothelial lineage).

Although our semi-quantitative RT-PCR showed that the level of expression of endothelial cell markers in THP-1 and RAW cells infected with PTN sense strand is similar to the positive control endothelial cells, we used an additional approach, real-time PCR (TaqMan), which is more sensitive and quantitative, to confirm the expression levels of selected endothelial cell markers in infected THP-1 cells. Using primers specific for VE-cadherin, vWf, and PECAM-1, we found that the expression levels of these endothelial cell markers in THP-1 cells infected with PTN sense strand (right panels, blue line) are similar to that of positive control endothelial cells (red line), but not in cells infected with the GFP control vector (yellow line). Similar results were obtained with Tie-2, and VE-cadherin (not shown). GAPDH amplification was used as an internal standard. These results confirm the semi-quantitative RT-PCR data and clearly show that the expression level of endothelial cell markers in PTN sense strand-infected THP-1 cells is similar to endothelial cells, suggesting that they may be biologically relevant.

We also used an immunohistochemical panel of endothelial cell markers (FLK-1, Tie-2, and VE-Cadherin) to determine the spatial distribution of the markers. The antibodies were obtained from Santa Cruz, Inc. and used at the recommended dilutions. Immunostaining was performed as we described previously (31). Immunohistochemical analysis of THP-1 cells infected with PTN sense strand showed strong expression of FLK-1 marker on the surface of cells (right panel). No expression

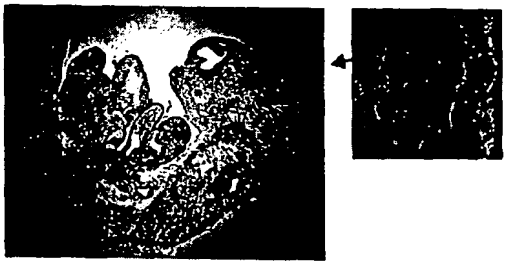


of FLK-1 was detected in THP-1 cells transduced with GFP control vector. Similar results were obtained with Tie-2, VE-Cadherin, and PECAM-1 (not shown).



We also examined the expression of another endothelial cell marker, $\alpha_v\beta_3$ integrin, in infected THP-1 cells. We selected this integrin because the interaction between $\alpha_v\beta_3$ integrin and extracellular matrix is crucial for endothelial cells sprouting from capillaries and for angiogenesis (32). In addition, $\alpha_v\beta_3$ integrin participates in the full activation of FLK-1 which is known to be important in tumor angiogenesis, inflammation, and tissue regeneration (32). Using a 1:100 dilution of $\alpha_v\beta_3$ antibody (obtained from Chemicon Co) and FACS analysis, we found that 82% of positive control human endothelial cells expressed $\alpha_v\beta_3$ integrin (not shown). Similarly, 88% of THP-1 cells infected with PTN sense strand expressed $\alpha_v\beta_3$ integrin (right panel, Sense). In contrast, less than 1% of THP-1 cells infected with GFP control retrovirus expressed $\alpha_v\beta_3$ integrin (right panel, GFP). We conclude that PTN induces signaling events in THP-1 cells that are important for endothelial cell activity and survival.

As a final in vitro study, we investigated the ability of the transduced THP-1 and RAW cells to form tubular structures. PTN- and GFP-transduced cells were cultured on three-dimensional fibrin matrices in RPMI/10% serum media. The fibrin gel was prepared essentially as described (33). Uninfected THP-1 and RAW cells were used as negative controls and human endothelial cells were used as a positive control. After 3 days in culture, cells infected with PTN sense strand invaded the fibrin matrix and started to form network-like structures in the three-dimensional gel, similar to positive control endothelial cells (not shown). In contrast, uninfected THP-1 and RAW cells as well as cells infected with the control GFP vector remained on top of the fibrin matrix and no network-like structure could be observed (not shown). These data demonstrate that infection of monocytic cells with PTN confers ability to the cells to rearrange in the fibrin gel with extended cytoplasm and interacting with surrounding cells, similar to endothelial cells.



Based on the outcome of the in vitro experiments, we decided to study the potential of the PTN-infected cells to be incorporated into the newly formed vasculature using a chick/mouse

chimeric assay. We injected 1-2 x10^5 cells in 2-4 μl into the hearts of stage 16-17 chick embryos with glass needles. Embryos were killed 2-3 days after injection, fixed, embedded in OTC, and frozen sections were cut and stained with anti-GFP antibodies (from Santa Cruz). As shown in the right panel, most of the GFP signals (brown color) appeared along blood vessels in the head, eyes, heart, and intersomitic region, and in some cases, forming a network structure 2-3 days after injection. High-resolution confocal image showed the association of GFP with microvessels (inset) around the eye (arrow). Tie-2 staining pattern was identical to the GFP staining pattern (not shown).

Objectives:

Aim 1) Does PTN induces transdifferentiation of breast cancer cells?

This aim will expand our preliminary data by asking whether the transdifferentiation activity of PTN is restricted to monocytic cells or PTN can also induce breast cancer cells to transdifferentiate into endothelial-like cells. We have found that PTN converts monocytic cells into endothelial-like cells. Based on this finding, we hypothesized that PTN is a plastogenic factor that promotes tumor neovascularization through transdifferentiation of cells into endothelial-like cells. To test this idea, we will express PTN in breast cancer cells and determine the phenotypic characteristics of infected cells.

Choudhuri et al (34) overexpressed PTN in MCF-7 breast carcinoma cells. Although the authors did not investigate the phenotypic modulation of cells by PTN, they showed that PTN-infected MCF-7 cells produced tumors that grew significantly faster than uninfected cells or cells transfected with a control DNA plasmid. Furthermore, these PTN-induced tumors had a greater vascular density compared to control tumors. In a corneal angiogenesis assay, it was shown that corneas receiving PTN-infected MCF-7 cells scored a higher angiogenic response when compared to using uninfected cells. It was found that the vascular pattern in the PTN-expressing tumors was strikingly different from those expressing VEGF. While MCF-7 cells expressing VEGF elicited an angiogenic response within 48 h (35), those expressing PTN required 2 weeks (34). We suggest that this relatively long incubation time required to induce angiogenesis by PTN-infected MCF-7 cells may be related to phenotypic alteration of MCF-7 cells into endothelial-like cells. This aim will explore this possibility.

We will overexpress PTN in hormone-dependent MCF-7 and ZR-75-1 cells as well as hormone-independent MDA-MB-231 breast cancer cell lines in order to investigate: 1] the

occurrence of the transdifferentiated endothelial phenotype in these cells, and 2] determine the effect of hormone on the transdifferentiation. We have already generated bicistronic retroviruses and all the necessary reagents for the proposed experiments. To save space, we will not describe the infection methodologies as they are described in detail in the Background Section. Cells will be transduced with the bicistronic retrovirus harboring PTN sense or anti-sense strands. Control cells will be infected with the GFP reporter vector. The infected cells will be examined for the expression of endothelial markers as described above. The ability of cells to form tumor in vivo will be assessed by standard xenograft assay in mice.

Aim 2) What is the active domain of PTN?

This aim will extend the preliminary data by asking which segment or domain of PTN molecule is responsible for its transdifferentiation activity. Prior studies have utilized a series of constructed PTN mutant proteins to determine the domains required for the transformation activity (36). It was found that a combination of 2 PTN segments was required for the transformation activity. However Inui et al (37) showed that only one segment corresponding to the N-terminal domain of PTN was required for transformation activity of PTN. Others have shown that while C-terminal end of PTN was required for transformation activity, the N-terminal segment retained its neurite outgrowth activity (38), suggesting that mitogenic and neurite outgrowth activities are mediated through different pathways. Employing a similar strategy, we will construct a series of PTN mutants and test them for their capacity to transdifferentiate THP-1 and RAW cells in order to identify the domain(s) responsible for biological activity.

The PTN mutants will be constructed with consideration to (i) the N- and C-terminals which contain heparin-binding β-sheet domains (39) and (ii) the middle portion that is a flexible linker between the terminal ends and is associated with the transformation activity (36). We have already cloned full-length human PTN. We will design PCR primers corresponding to the N-terminal and C-terminal half of the protein. Using the full-length PTN as a template, we will amplify the C-terminal and N-terminal domains of PTN and the veracity of nucleotide sequence will be verified by DNA sequencing. The PCR products will be cloned into the TOPO PCR-2 vector (Invitrogen), followed by subcloning into a retroviral bicistronic vector that we have developed (discussed above), using standard molecular biology techniques. As described above, the retroviral vectors will be packaged, viral titers will be determined, and monocytic cells will be infected with the viruses. The expression of endothelial markers will be assessed as discussed

above using PCR and immunohistochemical staining. Endothelial cell markers (e.g., FLK-1, Tie-2, VE-cadherin, PECAM-1, endothelial nitric oxide synthase and the von Willebrand factor) will be utilized to determine the extent of transdifferentiation in vitro. The ability of infected cells to promote neovascularization in vivo will be assessed by standard xenograft transplantation experiments. Infected THP-1 or RAW cells will be injected subcutaneously at a unique site in mice. Tumor size will be measured twice a week, starting from the second week following injection. Mice will be sacrificed 6 weeks after injection.

Aim 3) What is the downstream signaling in PTN-mediated transdifferentiation?

This aim will extend the effort of aim 2 by investigating the PTN-induced signaling in monocytes/macrophages. Currently, nothing is known about PTN signaling in monocytes/macrophages. As an initial step to understand PTN signaling involved in transdifferentiation of monocytes/macrophages to endothelial cells, we will concentrate our effort on the mitogen-activate protein (MAP) kinase pathway for two reasons: 1] this pathway is known to be activated by PTN in bovine epithelial lens cells (7), and 2] MAP kinase pathway is thought to be a key signaling pathway that have been implicated in phenotypic outcome for the endothelial cells and angiogenesis (40). Activation of MAP kinase pathway has been investigated in a variety of macrophages including THP-1 (41-45) and RAW (42, 46-48) cells. We will use inhibitors of MAP kinase to block the transdifferentiation of THP-1 and RAW cells transduced with PTN retroviruses. Control cells will include uninfected cells and cells infected with retroviruses carrying only the GFP reporter gene. After infection with the retroviruses, cells are allowed to establish an efficient level of transduction for 24-48 hours. Cells will then be treated with inhibitors of MAP kinase which known to be effective in THP-1 and RAW cells such as SB203580 (49) (Calbiochem, San Diego, CA). After treating for various time points, the expression of endothelial cell markers will be assessed, as we described above. To confirm the activation of the MAP kinase signaling pathway in cells undergoing transdifferentiation, we will perform established Western blot analysis to assess phosphorylation of specific protein substrate targets (e.g. SHC, ERK1, ERK2, and Akt1) in the PTN-transduced cells. Control cells that are not undergoing transdifferentiation should display lower levels of these phosphorylated proteins. We predict that the MAP kinase inhibitors will block the transdifferentiation activity of PTN, suggesting that this pathway is involved in PTN-mediated transdifferentiation of monocytes/macrophages into endothelial-like cells. Alternatively, it is possible that the

transduced THP-1 and RAW cells have already committed to the endothelial cell phenotype during the initial period of retroviral transduction and therefore, the inhibitors remain ineffective. To address this issue, we will add the inhibitors just prior to retrovirus infection in an attempt to block the transdifferentiation from proceeding. It is also possible that other pathways are responsible for PTN-induced signaling in monocyte/macrophages. As an initial step to explore such possibility, we will focus on the activation of Src which as been postulated as an intermediary signaling molecule for PTN activity (50).

Innovation: It is generally thought that tumor neovascularization occur through angiogenesis. According to this paradigm, angiogenic factors released by tumor cells or infiltrating macrophages promote proliferation of pre-existing endothelial cells or recruitment of endothelial progenitor cells. Recent findings on the plasticity of differentiated cells suggest a third unique possibility: that endothelial cells can be generated through transdifferentiation of monocyte/macrophages. In support of this hypothesis, we have identified PTN as a plastogenic factor that promotes transdifferentiation of monocytes/macrophages into endothelial cells. The finding that the endothelial cells required for blood vessel formation need not originate from either pre-existing endothelial cells or their precursor, but rather are generated de novo from other cell types provides a new target to be used to control tumor growth and metastasis.

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EXAMPLE 4

We recently discovered unique gene expression profiles of coronary and mammary arteries. While coronary arteries are prone to develop angiogenic sprouting and consequently, develop atherosclerotic plaques, internal mammary arteries are remarkably resistant to plaque formation. Using suppressive subtraction hybridization, we have identified genes that are differentially expressed in the porcine coronary and mammary arteries. We found that coronary artery strongly expressed pleiotrophin (PTN), a pro-angiogenic gene, whereas mammary artery does not express this gene [Qin, 2003 #3127].

PTN is normally expressed during embryogenesis, but rarely in healthy adult tissue. In the brain, PTN is expressed during embryonic development (see Fig. 9) and its expression is down-regulated in the adult brain. However, it is re-expressed in regions with high levels of neovascular formation of glioblastomas and the adult ischemic brain. In addition, PTN is produced by other human tumors such as breast cancer, pancreatic cancer, melanoma, meningioma, and neuroblastoma. Tumor cells transfected with dominant negative or ribozyme-targeted PTN and transplanted into nude mice yielded reduced tumor growth due to impaired angiogenesis whereas breast carcinoma cells overexpressing PTN have a growth advantage.

It is thought that PTN promotes tumor vascularization by stimulating endothelial cell recruitment, proliferation, and angiogenesis. We offer evidence that PTN may promote tumor vascularization through an entirely different mechanism that is independent of angiogenesis. Based on our findings, we propose that PTN promotes tumor vascularization, in part, by inducing transdifferentiation of monocytes/macrophages into endothelial cells. This study is designed to expand and extend this proposal.

Aim 1) Does PTN induces transdifferentiation of glioma cells into endothelial-like cells?

We have found that monocytes/macrophages can be converted into endothelial-like cells when stably transduced with retrovirus harboring PTN sense strand, but not PTN anti-sense strand or GFP control vector. We would now like to expand on these observations by asking whether this plastogenic activity of PTN is limited to monocyte/macrophages or whether tumor cells, such as glioma, can be coaxed by PTN to alter their phenotype into endothelial-like cells. To address this question, we will transduce glioma cells with the retrovirus harboring PTN and determine the phenotypic characteristics of the infected cells.

Aim 2) What is the active domain of PTN?

This aim will extend the preliminary data and effort of aim1 by asking which segment or domain of the PTN molecule is responsible for its transdifferentiation activity. Mapping of the active domain of PTN that is required for transdifferentiation of monocytic cells, or possibly glioma cells (aim 1), will help us to understand the molecular basis of PTN signaling. Prior studies have utilized a series of constructed PTN mutant proteins to determine the domains required for the transformation activity [Zhang, 1999 #3187]. We will construct a series of PTN mutants and test their ability to transdifferentiate monocytic cells.

Aim 3) What is the downstream signaling in PTN-mediated transdifferentiation?

This aim will extend the efforts of aims 1 and 2 by investigating the PTN-induced signaling. Currently, nothing is known about PTN signaling in monocytes/macrophages. As an initial step to understand PTN signaling involved in transdifferentiation of monocytes/macrophages to endothelial cells, we will concentrate our effort on the mitogen-activate protein (MAP) kinase pathway for two reasons: 1] this pathway is known to be activated by PTN in bovine epithelial lens cells [Souttou, 1997 #3086], and 2] MAP kinase pathway is thought to be a key signaling pathway that have been implicated in the phenotypic outcome of endothelial cells and angiogenesis [Lee, 2000 #3213]. Activation of MAP kinase pathway has been investigated in a variety of macrophages including THP-1 cells [Huang, 1999 #3200;McGilvray, 1998 #3198;Hambleton, 1996 #3199].

Background and significance

Vascularization of tumors is a highly complex process that is regulated by a balance between pro- and anti-angiogenic molecules [Carmeliet, 2000 #3101]. Pro- and anti-angiogenic molecules can emanate from cancer cells, endothelial cells, inflammatory cells, stromal cells, blood and the extracellular matrix. Three models have been proposed to explain tumor vascularization: angiogenesis, postnatal vasculogenesis, and vascular mimicry.

Angiogenesis: According to this model, endothelial cells are derived from the pre-existing endothelium of blood vessels. Recruitment and proliferation of endothelial cells is regulated by angiogenic factors that emanate from tumor cells, infiltrating leukocytes, and stromal cells. Among the hematopoietic cells, macrophages are thought to be critical in promoting angiogenesis by: a] releasing potent angiogenic factors such as VEGF, bFGF, TGF-α,

TNF-α, and IL-8, which induce recruitment and proliferation of endothelial cells from pre-existing vessels [Brogi, 1993 #3099;Ramos, 1998 #3104;Seljelid, 1999 #3112], and b] modulating extracellular matrix remodeling required for new blood vessel formation through secretion of various metalloproteinases (see Ono [Ono, 1999 #3136] for review). Thus, macrophages influence every stage of neovascularization by a paracrine mechanism, i.e., releasing factors that promote recruitment and proliferation of endothelial cells.

Postnatal vasculogenesis: The angiogenic factors produced by tumor cells recruit endothelial cells from two sources: pre-existing mature endothelial cells and circulating endothelial progenitor cells. Mature endothelial cells can be recruited either from pre-existing blood vessels (angiogenesis) or from circulating endothelial cells. Normal adults have 2.6±1.6 circulating endothelial cells per mm^3 of peripheral blood with most of these cells being quiescent and at least half being microvascular as defined by CD36 positivity [Solovey, 1997 #3058]. Circulating mature endothelial cells are detectable in pathological diseases marked by vascular injury conditions, such as sickle cell anemia, acute myocardial infarction, thrombotic thrombocytopenic purpura, and active cytomegalovirus infection [Solovey, 1997 #3058;Lefevre, 1993 #3059;Grefte, 1993 #3060;Hladovec, 1978 #3123;Hladovec, 1978 #3124].

The existence of circulating endothelial progenitor cells in adult humans as a characteristic feature of postnatal vasculogenesis has only recently been suggested. Asahara et al. [Asahara, 1997 #2897] isolated endothelial progenitor cells from adult human peripheral blood using magnetic bead selection of CD34+ hematopoietic cells. In vitro, majority of the primary adherent cells differentiated into spindle-shaped cells within 7-10 days of culture on fibronectin and expressed markers of endothelial cell characteristics. Kalka et al. [Kalka, 2000 #3163] used the primary adherence on fibronectin to isolate endothelial progenitor cells from total human peripheral blood mononuclear cells and also demonstrated the appearance of cells with an endothelial phenotype at a very high frequency after 7-10 days of culture. Animal models of ischemia and tumor growth demonstrated the contribution of endothelial progenitor cells to active neovascularization [Kalka, 2000 #3163;Asahara, 1999 #2898;Takahashi, 1999 #3067]. Shi et al. [Shi, 1998 #2904] and Nieda et al. [Nieda, 1997 #3164], using CD34+ cells at a much higher purity (>93%) than Asahara et al. [Asahara, 1997 #2897], observed adherent endothelial cell colonies. Since human peripheral blood was used as a stating point to isolate endothelial progenitor cells in these studies, the potential contribution of contaminating cells in

the interpretation of the data remains unclear. The experiments described above on the characterization of endothelial progenitor cells used cell preparations with 7% [Shi, 1998 #2904;Nieda, 1997 #3164] to 86% [Asahara, 1997 #2897] impurities, raising concerns about the origin of endothelial cells. Since endothelial progenitor cells have been isolated from the human peripheral blood mononuclear cell fraction containing varying degrees of monocytic cell contaminants that have a high capacity to adhere to extracellular matrix at the time of isolation, it is conceivable that monocytic cells, or its subpopulation, may be the source of endothelial progenitor cells. We offer evidence that support this notion (see preliminary data).

In addition to circulating mature and immature endothelial cells, transdifferentiation of bone marrow stem cells represents another source of endothelial cells. Several studies have suggested the existence of multipotent adult stem cells that have the potential to replenish several cell lineages in various tissues, even across the germ layer barrier [Orkin, 2000 #3053]. Adult hematopoietic cells are defined by their ability to self-renew while functionally repopulating the hematopoietic compartment for the lifetime of an individual. Like other tissue-specific stem cells, hematopoietic stem cells could retain plasticity capable of regenerating multiple cell types in non-hematopoietic tissues, including the endothelial cells. Multipotent adult mesenchymal stem cells also differentiate into many specialized cell types in culture and contribute to a wide range of developing tissues when injected into mouse blastocysts. When transplanted into adult mice, they engraft and differentiate into hematopoietic cells, epithelial cells and endothelial cells [Jiang, 2002 #3054].

Similar concerns about the role of cell contaminants have been raised in the interpretation of engraftment and differentiation of stem cells [Goodell, 2001 #3166]. While recent studies with purified hematopoietic stem cells indicate that, at least in some cases, stem cells or their progeny can transdifferentiate into nonhematopoietic cells, a definitive proof of transdifferentiation is still lacking, mainly due to cell populations, rather than a single cell, being used in the experiments. Preparations of a few thousand, or even few, purified hematopoietic stem cells could still include progenitors of other tissues as contaminants, raising the possibility that the grafts could be heterogeneous and therefore, the probability of two different types of cells contributing to two different lineages cannot be excluded. We offer evidence that human and mouse monocytic cell lines that represent homogeneous population of cloned cells can be induced to transdifferentiate into endothelial-like cells (see preliminary data).

Vascular mimicry: Some tumors are vascularized without significant angiogenesis, by forming vascular channels on their own through a non-endothelial cell process [Maniotis, 1999 #3168]. For example, 15% of blood vessels in xenografted and spontaneous human colon carcinomas are mosaic in nature [Chang, 2000 #3260]. It has been suggested that highly invasive primary and metastatic melanoma cells may generate vascular channels, lined externally by melanoma cells themselves, and facilitate tumor perfusion independent of angiogenesis [Folberg, 2000 #3169]. It is reported that tumor cells migrate toward existing host organ blood vessels in sites of metastases, or in vascularized organs such as the brain, to initiate blood vessel-dependent tumor growth as opposed to classic angiogenesis [Holash, 1999 #3170]. It is an open question whether these vessels result from cancer cells invading the vessel lumen, cancer cells mimicking endothelial cells, co-opted vessels or apoptosis of endothelial cells which exposes underlying cancer cells.

In summary, it is thought that tumor angiogenesis starts only when the neoplastic mass reaches 1 mm in diameter and when hypoxia occurs and that it is essentially mediated by angiogenic molecules elaborated by tumor cells and infiltrating leukocytes. Nevertheless, the mechanisms responsible for vascularization of in situ tumors and their remodeling are still poorly understood. Three models have been proposed to describe vascularization of tumors with differences based on the origin of blood vessel cells. The new capillary can be formed by endothelial cells derived from either pre-existing blood vessels (angiogenesis), or endothelial progenitor cells (postnatal vasculogenesis). Besides endothelial cells, blood vessel walls can be lined with cancer cells alone or a mosaic of cancer and endothelial cells (vascular mimicry). We offer evidence for the existence of a new model for capillary formation. This new model is based on the transdifferentiation of monocytes/macrophages, or possibly tumor cells, into endothelial cells that could subsequently contribute to tumor vascularization.

Preliminary Studies

Hypothesis:

Recent evidence suggests that the ability of immature cells to differentiate into different cell types is not an exclusive feature of progenitor cells. Rather, post-mitotic differentiated cells or differentiation-committed cells can also undergo transdifferentiation. For example, hepatic oval cells could be re-differentiated to hormone-producing pancreatic islet cells by culturing in

high-glucose medium [Yang, 2002 #3184]. Conversely, exposure of a pancreatic cell line to glucocorticoid resulted in their transdifferentiation to hepatic cells [Shen, 2000 #3135]. In a co-culture model, neurosphere-derived cells can differentiate into skeletal muscle cells in the presence of differentiating myoblasts [Rietze, 2001 #3185]. Finally, injection of 3T3-L1 preadipocyte cells into the peritoneal cavity of nude mice converted the cells into macrophages [Charriere, 2003 #3134]. These studies suggest that differentiation is an ongoing process that can be modified by key regulators. Factors that regulate a cell's plasticity remain largely unknown.

Since both endothelial and hematopoietic cell lineages share a common precursor during embryogenesis (hemeangioblasts) and committed cells are capable of switching their fate, we reasoned that monocytes could be converted into endothelial cells. Specifically, we hypothesize that angiogenic factors may promote neovascularization by inducing transdifferentiation of infiltrating monocytes into endothelial-like cells. A corollary hypothesis would hold that angiogenic factor may behave as a plastogenic factor.

We recently discovered a unique gene expression profile of coronary and mammary arteries. While coronary arteries are prone to develop angiogenic sprouting and consequently, develop atherosclerotic plaques, internal mammary arteries are remarkably resistant to plaque formation. We hypothesized that the normal coronary artery has an environment that is conducive to angiogenic processes. In contrast, the normal internal mammary artery milieu counters this event. We used suppressive subtraction hybridization (SSH) to generate reciprocal cDNA collections of representing mRNA specific to porcine coronary vs. porcine mammary arteries. We screened 1000 SSH cDNA clones by dot blot array and sequenced 600 of these showing the most marked differences in expression. Northern blot and in situ hybridization confirmed the differential gene expression pattern identified by the dot blot arrays.

We found that pleiotrophin (PTN) is strongly expressed in the pro-angiogenic coronary artery, but not in the angiogenic-resistant internal mammary arteries [Qin, 2003 #3127]. PTN is a 18-kDa protein that contains 24% basic residues (18% lysines), arranged mainly in two clusters at the N- and C-terminal regions, and five intra-chain disulfide bonds (for general discussions see Muramatsu [Muramatsu, 2002 #3021]). The molecule is organized in two β-sheet domains linked by a flexible linker with each of these domains having a heparin-binding site. At least one heparin-binding site is involved in the dimerization of this growth factor that is important for

PTN mitogenic activity since this activity on BEL cells is modulated by exogenous addition of glycosaminoglycans [Vacherot, 1999 #3259]. Furthermore, treatment of BEL cells with heparinase III abolished PTN mitogenic activity, which could be restored by the addition of soluble heparin.

PTN is mitogenic for various cell types, promotes angiogenesis, stimulates neurite outgrowth from cultured neurons, and induces cell migration. PTN is normally expressed during embryogenesis, but rarely in healthy adult tissues [Iwasaki, 1997 #3215]. However, PTN is produced by some human tumors including breast cancer [Zhang, 1997 #3179], pancreatic cancer [Weber, 2000 #3216], melanoma [Czubayko, 1996 #3178], meningioma [Mailleux, 1992 #3039] and neuroblastoma [Nakagawara, 1995 #3041]. Tumor cells transfected with dominant negative or ribozyme-targeted PTN and transplanted into nude mice yielded reduced tumor growth due to impaired angiogenesis [Czubayko, 1996 #3178;Zhang, 1997 #3179;Weber, 2000 #3216] whereas breast carcinoma cells overexpressing PTN have a growth advantage [Choudhuri, 1997 #3193]. Thus, various tumor cells produce the angiogenic factor PTN.

PTN expression in the brain occurs mainly during embryonic and early postnatal periods, but not in the adult brain [Bloch, 1992 #3220]. PTN is re-expressed in glioblastoma and in adult rat brain after acute ischemia. In high-grade gliomas, PTN mRNA or protein is detectable and its expression is confined to proliferating cells in situ [Mentlein, 2002 #3219]. It is thought that PTN produced by gliomas contributes to their malignancy by targeting endothelial cells [Mentlein, 2002 #3219]. In the ischemic brain, PTN expression is concentrated within areas of exuberant neovasculature that formed at the margins of the infarct and in macrophages around the newly formed vessels [Takeda, 1995 #3080;Yeh, 1998 #3042]. It has been suggested that PTN produced by macrophages promote brain angiogenesis by stimulating recruitment of endothelial cells [Takeda, 1995 #3080;Yeh, 1998 #3042].

Macrophages are thought to be critical in promoting angiogenesis by releasing potent angiogenic factors that stimulate recruitment and proliferation of endothelial cells from preexisting vessels [Brogi, 1993 #3099;Ramos, 1998 #3104;Seljelid, 1999 #3112] and by modulating extracellular matrix remodeling required for new blood vessel formation through secretion of various metalloproteinases (see Ono [Ono, 1999 #3136] for review). Thus, macrophages influence every stage of neovascularization by a paracrine mechanism, i.e., releasing factors that promote recruitment and proliferation of endothelial cells.

In addition to the effects on neighboring cells, we hypothesized that angiogenic factors produced by macrophages affect activity of monocytes/macrophages in an autocrine fashion. Since, PTN expression is concentrated within areas of exuberant neovasculature and co-localized with macrophages in the ischemic brain [Takeda, 1995 #3080;Yeh, 1998 #3042], we asked whether macrophages express PTN, and if so, what effects does this expression have on macrophage activity. There is no published report on the activity of PTN on macrophages.

Expression of PTN by monocytic cells

In preliminary experiments, we first determined whether PTN is expressed by monocytes/macrophages. In addition, since activated tumor-associated macrophages express TNF-α [Ono, 1999 #3136], a known potent angiogenic factor, we asked whether TNF-α regulates expression of PTN in macrophages. We treated exponentially growing cultured human THP-1 and mouse RAW monocytic cells (obtained from ATCC) with TNF-α (in RPMI/10% serum) for 6 h. Control cells were maintained in RPMI/10% bovine serum. In addition, we used exponentially growing human fibroblastic cells (DMEM/10% serum) for comparison. RNA isolation and Northern blot was performed essentially as we described [LaFleur, 1994 #462]. Briefly, cultured cells were lysed with guanidine thiocyanate and RNA was isolated by centrifugation through a cesium chloride cushion. The RNA was quantified and 10 μg/lane was analyzed by electrophoresis followed by blotting. The blot was probed with the PTN cDNA probe (top panel). To control for loading, the blot was re-probed with β-actin (lower panel).

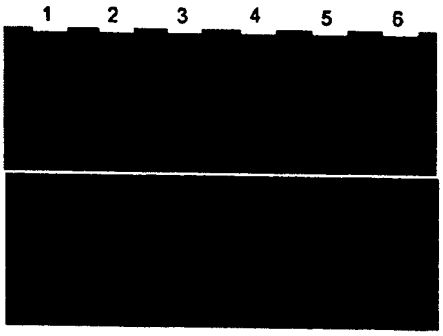


Fig. 1. Expression of PTN by activated monocytic cells.

Northern blot analysis revealed that untreated, exponentially growing cultured human THP-1 (Fig. 1, right panel, lane 1) and mouse RAW (lane 4) cells do not express PTN mRNA. Addition of 10 ng/ml of TNF-α markedly up-regulated expression of PTN in human THP-1 cells (lane 2), and in mouse RAW cells (lane 3). Exponentially growing cultured human fibroblasts did not express PTN (lane 5) and addition of TNF-α had no effect (lane 6). These data demonstrate that: 1] the expression of PTN in macrophages is regulated by TNF-α and 2] the signaling events activated by TNF-α in the expression of PTN is conserved in human and mouse. These data suggest that TNF-α activity in

macrophages may be mediated, in part, by PTN.

To further test our hypothesis of the role of PTN in macrophage-mediated angiogenesis, we have three options: 1] Add TNF-α to cells and examine the effect. This would not be a good idea because TNF-α is a pleiotrophic factor that has many known and unknown effects on macrophages, which would complicate interpretation of the data. 2] Add recombinant PTN to monocytic cells and evaluate the outcome. This is also not a good approach because recombinant PTN produced in baculovirus, yeast, or E. coli does not retain the full-range of activities and the recombinant protein produced in mammalian cells is impure and losses its activity when purified [Fang, 1992 #2530]. 3] Therefore, to test our hypothesis, we decided to clone the full-length human PTN cDNA and generate stably transfected THP-1 and RAW monocytic cells using a bicistronic retrovirus vector.

Cloning of human PTN gene

We used the full-length human PTN open reading frame (accession # NM_002825) to clone the full-length cDNA. The PTN cDNA was generated by reverse transcription of human brain polyadenylated mRNA (Clontech) and amplification via polymerase chain reaction with specific primers for PTN (5'AAAATGCAGGCTCAACAGT AND 5'TGTTTGCTGATGTCCTTT). The PCR product was cloned into pCRII-TOPO vector (Invitrogen) and five clones were selected for further analysis. Nucleotide sequence analysis of the clones revealed that they contain full-length PTN cDNA (3 clones of sense and 2 clones of anti-sense orientations). To further validate the sequence veracity, it was electronically translated into protein using the ExPASY Translate tool program and its molecular weight was determined by the ExPASY Compute pI/Mw tool program. The theoretically translated product was composed of 136 amino acids with a theoretical molecular weight of 15.3 kDa and a pI=10.3. We concluded that the cloned cDNA sequence matched the full-length PTN nucleotide and amino acid sequences found in the GenBank database. After feeling confident about the cloned PTN gene, we subcloned the gene into a retroviral vector in order to transduce monocytic cells.

Generation of retroviral vectors, retrovirus production, and infection of monocytic cells

We constructed a bicistronic retroviral vector for our experiments. The bicistronic retroviral vector was constructed using the pLP-EGFP-C1 plasmid (Clontech) containing the enhanced green fluorescent protein (GFP) gene under the control of the CMV promoter. PTN cDNA was positioned down-stream to the CMV promoter. Next, we cloned an internal ribosomal entry site (IRES) sequence downstream of PTN and upstream of GFP in order to generate the bicistronic retroviral vector (Fig. 2). The IRES in this vector permits simultaneous expression of PTN and GFP from one mRNA. This bicistronic retroviral vector has several advantages over monocistronic vectors: 1] it allows us to follow PTN gene expression in infected cells *in vitro* and *in vivo* by monitoring for GFP expression, 2] PTN translation occurs independent of GFP allowing for the secretion of PTN from cells while GFP remains in the cells, and 3] transduced cells can be isolated by FACS.

Figure 2. Structure of the retroviral expression vector containing the human PTN cDNA. The pLP-GFP-C1 retroviral expression plasmid was modified with the insertion of human PTN cDNA (first cistron) and an IRES sequence between the CMV promoter and the GFP (second cistron). The first gene was positioned either in a sense or anti-sense orientation after the CMV promoter.

All retroviral expression plasmids were constructed using the pLP-C1-IRES-GFP (Clontech) retroviral vector and standard molecular biology techniques. In these cassettes, transcription is initiated by promoter sequences within the viral 5' long terminal repeat (LTR) and terminated by polyadenylation sequences within the 3' LTR. Translation of the first (PTN) and second (GFP) cistrons from a single mRNA proceeds by ribosome binding to 5' Cap and IRES sequences, respectively. The full-length cDNA of human PTN was cloned into the *BamH/NotI* sites of pLP-C1-IRES-GFP. The retrovirus was packaged using a 293 packaging cell line provided by Clontech. After transfection of packaging cells, the medium was changed at 10 h and again at 24 h after transfection. Virus collected between 24 and 48 h after transfection was used for infection. Retroviral titers between $1x10^6$ and $2x10^7$ cfu/ml were determined by limiting dilution with NIH3T3 cells. For infection, $4x10^5$ human THP-1 or mouse RAW monocytic cells were plated in 25-cm^2 flasks 24 h before infection in normal growth medium (DMEM/10%FBS) to obtain exponentially growing cultures. The medium was replaced with 4 ml of retroviral supernatant (approximate MOI 2.5-25 cfu/cell) supplemented with 4 pg/ml polybrene. After 12 h, retroviral supernatants were removed and replaced with fresh normal medium for 48 h. Under these conditions, no apparent toxicity was observed after a 12-h exposure to retroviral supernatants containing polybrene in cultured cells.

Next, we asked whether GFP reporter gene expression could be used to isolate distinct

populations of PTN-expressing cells and if gene expression is maintained over multiple passages in culture. Cultured monocytic cells were infected with PTN-IRES-GFP virus or control IRES-GFP virus. Polyclonal populations of cells ($3x10^5$ each) expressing low or high levels of the GFP reporter gene were then isolated by flow cytometry. Immediate post-sort analysis confirmed the isolation of distinct populations of cells based on GFP expression (not shown). Cells were subcultured for an additional passage and analyzed for expression of the GFP reporter gene by flow cytometry and for expression of PTN by Western blot analysis (see below). Sorted cells maintained their relative expression levels of GFP, and there was excellent correlation between GFP and PTN expression. We concluded that GFP reporter gene expression can be used to isolate distinct populations of cells expressing different levels of PTN by flow cytometry.

As shown previously in Fig. 1, human THP-1 and mouse RAW monocytic cells do not express PTN under basal condition and activation of monocytic cells by TNF-α was required for the expression of PTN. Therefore, we used Northern and Western blot analyses to investigate expression of PTN in the infected monocytic cells. Consistent with data in Fig. 1, we found that unactivated monocytic cells do not express PTN mRNA; however, cells infected with PTN sense and anti-sense strand expressed PTN mRNA (not shown). PTN mRNA was not detected in the cells infected with the GFP control vector. Western blot analysis revealed that PTN gene product is expressed by THP-1 or RAW cells that have been infected with PTN sense strand, but not infected with PTN anti-sense strand or control GFP vector (not shown). After feeling confident about the expression of PTN by monocytic cells, we asked what is the role of PTN in macrophage-mediated angiogenesis.

What is the role of PTN in macrophage-mediated tumor neovascularization?

As discussed above, based on close embryological association between endothelial cells and monocytes and the ability of differentiated cells to switch their fate, we reasoned that monocytes could be converted into endothelial cells. Specifically, we hypothesize that PTN promotes transdifferentiation of infiltrating monocytes into endothelial-like cells thus stimulating tumor neovascularization. A corollary hypothesis would hold that PTN is a plastogenic factor.

To test our hypothesis, we examined the expression of several established endothelial cell markers in PTN-infected cells. The markers were: vascular endothelial growth factor receptor-2 (Flk-1)[Yamaguchi, 1993 #3144], Tie-2[Sato, 1993 #3049], vascular endothelial-cadherin (VE-cad)[Lampugnani, 1992 #3142], PECAM-1[DeLisser, 1994 #3141], endothelial nitric oxide synthase (eNOS)[Moncada, 1991 #3146], the von Willebrand factor (vWf)[Moncada, 1991 #3146], and CD34 [Young, 1995 #3143]. Total cellular RNAs were isolated by using RNEasy Mini Kit (Qiagen, Chatsworth, CA). RT-PCR was performed using Qiagen OneStep RT-PCR kit with the addition of 10 units Rnase inhibitor (GIBCO/BRL) and 40 ng RNA. The primer sequences and PCR conditions are included in the appendix. Primer sequences for each specific marker were designed using human DNA sequences from the GenBank database. To ensure semi-quantitative results of the RT-PCR assays, the number of PCR cycles for each set of primers was checked to be in the linear range of the amplification. In addition, all RNA samples were adjusted to yield equal amplification of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as an internal standard. The amplified products were separated on 1.2% agarose gels with ethidium bromide (E-Gel, Invitrogen).

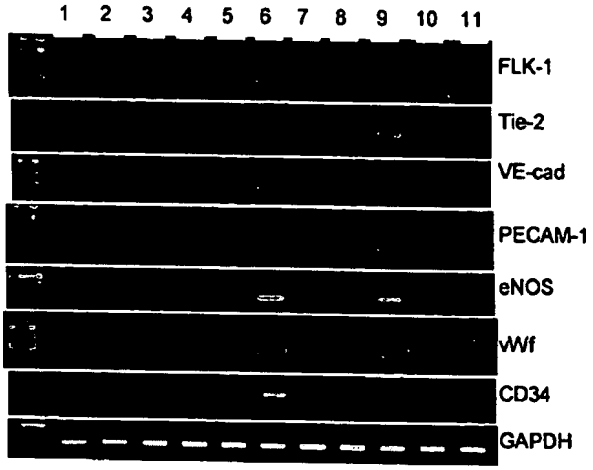


Fig.3. RT-PCR analysis of endothelial cell marker expression. Total RNA was isolated from various cell types and subjected to RT-PCR analysis. The PCR conditions were shown in the appendix. The GAPDH was used as an internal standard.

Infected THP-1 cells and uninfected monocytic cells were analyzed by semi-quantitative PCR for the expression of endothelial cell markers. As a comparison, the expression pattern of these markers was analyzed in non-monocytic NIH 3T3 cells, human smooth muscle cells, and human skin fibroblasts. All cell lines were obtained from ATCC and cultured according to their instructions. The analysis from three independent experiments is shown in Fig. 3. THP-1 cells infected with PTN sense strand readily expressed detectable levels of endothelial cell markers (lane 9), similar to that of positive control human coronary artery endothelial cells (lane 6). In contrast, the expression of these markers was not detectable in uninfected mouse monocytic RAW cells (lane 1), human promonocytic leukemic U937 cells (lane 2), and THP-1 cells (lane 3). Similarly, these markers were not detectable in NIH 3T3 cells (lane 4), human smooth

muscle cells (lane 5), RPMI 8226 B lymphocyte plasmacytoma cell line (lane 7), and human skin fibroblasts (lane 8). In addition, these endothelial cell markers were not detected in either THP-1 cells infected with PTN anti-sense strand (lane 10) or GFP control vector (lane 11). The weak expression of FLK-1 and Tie-2 in smooth muscle cells (lane 5) is consistent with the previous report on the expression of these endothelial cell markers in human smooth muscle cells [Ishida, 2001 #3138]. The expression pattern of these endothelial cell markers in PTN-infected RAW cells was similar to PTN-infected THP-1 cells (not shown).

These data clearly demonstrate that uninfected monocytic and non-monocytic cells do not express endothelial cell markers. However, infection of THP-1 or RAW cells with PTN sense strand markedly up-regulates expression of established endothelial cell markers. In addition, since the number of PCR cycles for each set of primers was chosen to be in the linear range of the amplification, these data show that the expression levels of endothelial cell markers are similar to those of positive control endothelial cells. Further, PTN appears to up-regulate expression of endothelial cell markers that are representative of mature endothelial cells such as VE-cadherin, PECAM-1, eNOS, and VWF, suggesting that PTN-infected THP-1 cells assume a phenotype of mature endothelial-like cells.

Several studies have suggested the existence of multipotent adult stem cells that have the potential to replenish several cell lineages in various tissues, even across the germ layer barrier [Orkin, 2000 #3053]. It is thought that adult hematopoietic stem cells could retain plasticity and therefore, capable of regenerating multiple cell types in nonhematopoietic tissues, including endothelial cells [Jiang, 2002 #3132]. The monocytic cell lines that we have used in our studies are established cell lines with known monocytic cell characteristics; therefore, they do not have multipotent characteristics like adult hematopoietic stem cells. The results of PCR studies described above clearly support this and show that the uninfected cells do not express endothelial cell markers. However, to further examine whether the monocytic cells that we used have characteristics of stem cells or immature endothelial cells, we examined the expression of zinc finger transcription factors known to be expressed in immature and mature endothelial cells such as GATA-2 and GATA-3 [Lee, 1991 #3150;Gumina, 1997 #3151;Jahroudi, 1994 #3152;Cowan, 1998 #3153], as well as the expression of Oct-4 transcription factor known to be expressed in pluripotent cells [Yeom, 1996 #3139].

Semi-quantitative PCR analysis (Fig. 4) showed that THP-1 cells infected with PTN sense strand (lane 9) expressed these transcription factors and that the expression levels of the factors are similar to control human endothelial cells (lane 6). In sharp contrast, uninfected monocytic RAW, U937, and THP-1 cells (lanes 1-3) as well as THP-1 cells infected with either PTN anti-sense strand (lane 10) or GFP control vector (lane 11) did not express the transcription factors. In addition, non-monocytic NIH 3T3 cells (lane 4), smooth muscle cells (lane 5), RPMI 8226 B lymphocyte plasmacytoma cells (lane 7), and human skin fibroblasts (lane 8) also did not express GATA-2 and GATA-3.

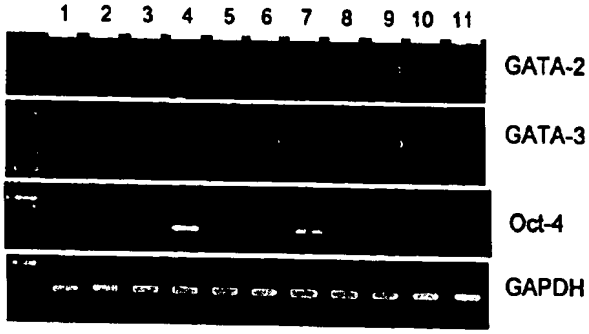


Fig.4. Expression of transcription factors. Total RNA was extracted from each cell type and RT-PCR was performed as described in Fig. 3.

We also used semi-quantitative PCR to investigate the expression of Oct-4 transcription factor. PCR analysis revealed that none of the monocytic cells examined expressed Oct-4, suggesting that they have a mature phenotype. Similarly, control non-monocytic cells such as smooth muscle cells, endothelial cells, and human fibroblasts did not express Oct-4. Interestingly, NIH 3T3 cells (lane 4) and RPMI 8226 B lymphocyte plasmacytoma cell line (lane 7) expressed Oct-4 suggesting that they have characteristics of immature cells.

The results of PCR experiments described above led us to hypothesize that PTN infection of THP-1 and RAW monocytic cells induces transdifferentiation of the cells into endothelial-like cells. We consider this process to be transdifferentiation because it meets the two criteria required for this event [Eguchi, 1993 #3043]: 1) The two types of differentiated states before and after transdifferentiation is clearly defined (monocytic cell lines vs. endothelial cells), 2) the cell types are of two different lineages (monocytic lineage vs. endothelial lineage).

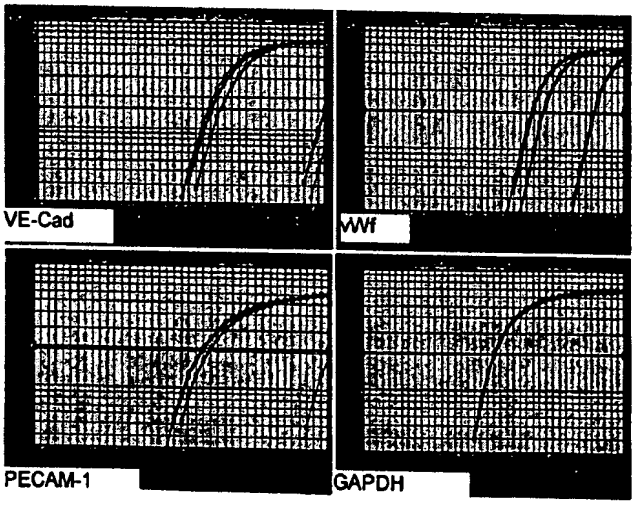


Fig. 5. Real-time PCR analysis of selected endothelial cell markers. GAPDH was used as an internal control.

Although our semi-quantitative RT-PCR showed that the level of expression of endothelial cell markers in THP-1 and RAW cells infected

with PTN sense strand is similar to the positive control endothelial cells, we used an additional approach, real-time PCR (TaqMan), which is more sensitive and quantitative, to confirm the expression levels of selected endothelial cell markers in infected THP-1 cells. Using primers specific for VE-cadherin, vWf, and PECAM-1, we found that the expression levels of these endothelial cell markers in THP-1 cells infected with PTN sense strand (Fig. 5, blue line) are similar to that of positive control endothelial cells (red line), but not in cells infected with the GFP control vector (yellow line). Similar results were obtained with Tie-2, and VE-cadherin (not shown). GAPDH amplification was used as an internal standard. These results confirm the semi-quantitative RT-PCR data and clearly show that the expression level of endothelial cell markers in PTN sense strand-infected THP-1 cells is similar to endothelial cells, suggesting that they may be biologically relevant.

We also used an immunohistochemical panel of endothelial cell markers (FLK-1, Tie-2, VE-Cadherin, PECAM-1) to determine the spatial distribution of the markers. The antibodies were obtained from Santa Cruz, Inc. and used at the recommended dilutions. Immunostaining was performed as we described previously [Wallner, 1999 #2319]. Immunohistochemical analysis of THP-1 cells infected with PTN sense strand showed strong expression of FLK-1 marker on the surface of cells (Fig. 6, right panel). No expression of FLK-1 was detected in THP-1 cells transduced with GFP control vector (left panel). Similar results were obtained with Tie-2, VE-Cadherin, and PECAM-1 (not shown).

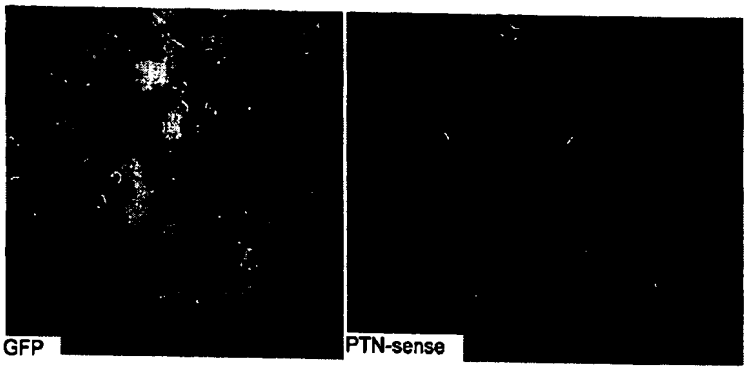


Fig. 6. Immunostaining analysis of PTN-infected THP-1 cells. Cultured THP-1 cells were fixed and then treated with by anti-FLK-1 antibody followed by secondary antibody. 20X magnification

To precisely determine the topographical relationship between distribution of transcription factors and endothelial cell markers in THP-1 cells infected with PTN sense strand, we performed double staining of the infected cells with anti-human GATA-2 rabbit polyclonal antibody (obtained from Santa Cruz) and anti-human VE-cadherin mouse monoclonal antibodies. A representative confocal image of THP-1 cells infected with PTN sense strand is

shown in Fig. 7. THP-1 cells infected with PTN sense strand and GFP control were cultured on coverslips and stained with anti-VE-cadherin monoclonal antibody. Afterwards, cells were permeablilized followed by staining with anti-GATA-2 rabbit polyclonal antibody (obtained from Santa Cruz). The secondary antibodies (Alexa Fluor 633 goat anti-rabbit antibody and Alexa Fluor 568 goat anti-mouse antibody) were used at 1:500 dilutions, as recommended by

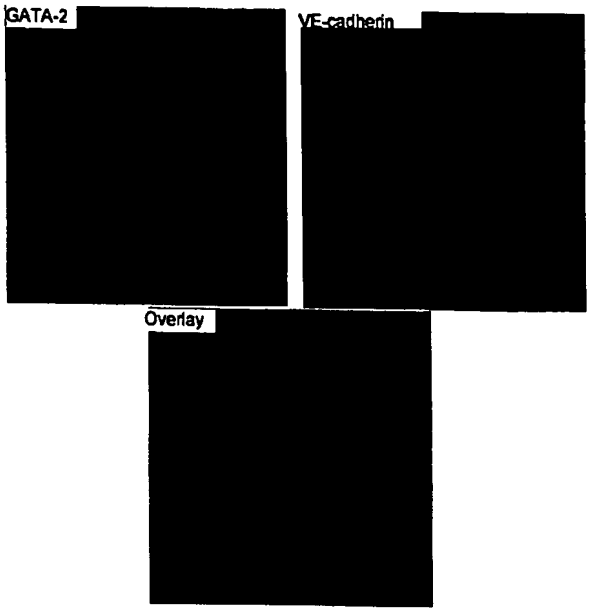
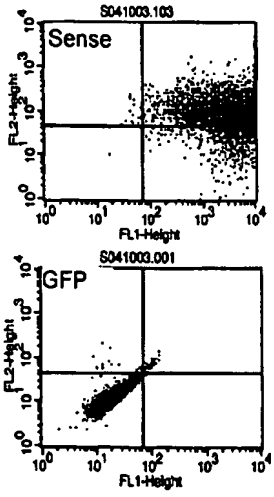


Fig. 7. Representative confocal image of THP-1 cells infected with PTN sense strand. The infected cells were permelized with 0.2% Tween 20 followed by staining with anti-VE-cadherin monoclonal antibody or anti-GATA-2 polyclonal antibody. The red color represents VE-cadherin and blue color shows GATA-2.

Molecular Probe. Consistent with the light microscopy results, we found strong expression of VE-cadherin on the surface of PTN-infected cells (red color). As expected, GATA-2 expression was concentrated in the nucleus (blue color). The overlay clearly showed the co-expression of two endothelial cell markers in THP-1 cells infected with PTN sense strand. No VE-cadherin or GATA-2 staining were detected in THP-1 cells infected with the control vector (not shown).

We also examined the expression of another endothelial cell marker, $\alpha_v\beta_3$ integrin, in infected THP-1 cells. We selected this integrin because the interaction between $\alpha_v\beta_3$ integrin and extracellular matrix is crucial for endothelial cells sprouting from capillaries and for angiogenesis [Soldi, 1999 #3181]. In addition, $\alpha_v\beta_3$ integrin participates in the full activation of FLK-1 which is known to be important in tumor angiogenesis, inflammation, and tissue regeneration [Soldi, 1999 #3181]. Using a 1:100 dilution of $\alpha_v\beta_3$ antibody (obtained from Chemicon Co) and FACS analysis, we found that 82% of positive control human



endothelial cells expressed $\alpha_v\beta_3$ integrin (not shown). Similarly, 88% of THP-1 cells infected with PTN sense strand expressed $\alpha_v\beta_3$ integrin (Fig. 8, right panel, Sense). In contrast, less than 1% of THP-1 cells infected with GFP control retrovirus expressed $\alpha_v\beta_3$ integrin (Fig. 8, right panel, GFP). We conclude that PTN induces signaling events in THP-1 cells that are important for endothelial cell activity and survival.

As a final *in vitro* study, we investigated the ability of the transduced THP-1 and RAW cells to form tubular structures. PTN- and GFP-transduced cells were cultured on three-dimensional fibrin matrices in RPMI/10% serum media. The fibrin gel was prepared essentially as described [Koolwijk, 1996 #3212]. Uninfected THP-1 and RAW cells were used as negative controls and human endothelial cells were used as a positive control. After 3 days in culture, cells infected with PTN sense strand invaded the fibrin matrix and started to form network-like structures in the three-dimensional gel, similar to positive control endothelial cells (not shown). In contrast, uninfected THP-1 and RAW cells as well as cells infected with the control GFP vector remained on top of the fibrin matrix and no network-like structure could be observed (not shown). These data demonstrate that infection of monocytic cells with PTN confers ability to the cells to rearrange in the fibrin gel with extended cytoplasm and interact with surrounding cells, similar to endothelial cells.

Based on the outcome of the *in vitro* experiments, we decided to study the potential of the PTN-infected cells to incorporate into the newly formed vasculature using a chick/mouse chimeric assay. We injected 1-2 x10^5 cells in 2-4 µl into the hearts of stage 16-17 chick embryos

Fig. 9. Contribution of RAW cells stably transduced with PTN to vascular formation *in vivo*. Frozen sections of chick embryos 3 days after injection with RAW cells expressing only GFP (panel a) or PTN sense strand plus GFP (b). The sections were stained with 1:100 dilution of anti-GFP antibody (Santa Cruz). Panel C shows high resolution confocal image of chicken embryo head.

with glass needles. Embryos were killed 2-3 days after injection, fixed, embedded in OTC, and frozen sections were cut and stained with anti-GFP antibodies. As shown in Fig. 9, the chicken embryo injected with RAW cells expressing only GFP did not stain with GFP antibody (panel a). In contrast, injection of RAW cells expressing both PTN and GFP (panel b) showed GFP staining (brown color) concentrated along blood vessels in the head, eyes, heart, and intersomitic region, and in some cases, forming a network structure 2-3 days after injection. Confocal image of the chicken embryo from panel b showed the association of GFP signal with microvessels in the head region (panel c). Tie-2 antibody staining pattern showed a similar pattern of staining (not shown).

In summary, our data clearly show that the uninfected monocytic cell lines that we have used do not have endothelial cell characteristics. The cells acquired endothelial cell markers when cells are transduced with retrovirus harboring PTN sense strand, but not infected with either PTN anti-sense strand or control GFP vector. Immunostaining data showed that the endothelial cell markers normally found on the surface of endothelial cells such as Tie-2, FLK-1, and VE-cadherin are expressed on the cell membrane of PTN-transduced cells indicating that the markers have correct topological configuration. The monocytic cells infected with PTN sense strand express $\alpha_v\beta_3$ integrin, suggesting that they have the potential to interact with extracellular matrix components that are found in the vasculature. The infected cells are also capable of forming tubular structure *in vitro* and found to be incorporated into newly formed vessel of developing chicken embryo, suggesting that they behave like endothelial cells both *in vitro* and *in vivo*. Overall, we offered evidence that PTN coaxed monocyte/macrophage lineage into endothelial-like lineage, suggesting that there may be merit in studying this event in more detail.

We would like to emphasize that our finding of endothelial cell generation from monocytic cells is clearly different from the other existing models of endothelial cell formation. Our finding is clearly different from an angiogenesis model where endothelial cells are derived from pre-existing vasculature. In addition, our finding is clearly different from a vasculogenesis model where endothelial cells originates either from maturation of hemeangioblasts or angioblasts. The THP-1 and RAW cell lines that we used are widely known differentiated monocytic cells that (i) do not express endothelial cell markers or (ii) express markers indicating that they do not have an endothelial progenitor cell phenotype. In addition, our finding is different from the vascular mimicry model proposed by Maniotis *et al.* [Maniotis, 1999 #3168]

where vascular channels are formed without the participation of endothelial cells and independent of angiogenesis. According to the vascular mimicry model, tumor cells themselves either carry blood or connect to the host's blood supply [Folberg, 2000 #3169]. These channel-forming tumor cells do not express endothelial cell markers [Weidner, 2002 #3223]. Our finding is different from the vascular mimicry model because the transdifferentiated monocytic cells express endothelial cell markers, unlike the cells that form blood channels in the model. Despite differences, there are some similarities between our finding and vasculogenesis or vascular mimicry models. Our finding shares some resemblance to the vasculogenesis model of blood vessel formation owing to the *de novo* nature of endothelial cell generation. Our finding also shares some similarity to the vascular mimicry model owing to the formation of endothelial cells without participation of either endothelial cells or its progenitor cells.

We concentrated on PTN as a potential plastogenic factor in part, because of recent finding described by Sugino *et al.* [Sugino, 2002 #3077]. They compared gene expression profiles of highly invasive metastatic murine mammary tumor cells with those of non-metastatic cells and found that pleiotrophin (PTN) is selectively up-regulated in the invasive metastatic cells. Although the expression of endothelial cell markers in these metastatic cells were not investigated, PTN expression was found to correlate with the ability of the metastatic cells to form vasculature and connect to blood vessels without participation of pre-existing blood vessel endothelial cells. However, we do not know whether this plastogenic activity is specific to PTN or other angiogenic factors have similar properties. In addition, we do not know whether other hematopoietic cells or tumor cells have the potential to transdifferentiate into endothelial-like cells by PTN or that the effect is restricted to monocytic cells. Regardless of these issues, the finding that the endothelial cells required for blood vessel formation need not originate from either pre-existing endothelial cells or their precursors, but rather are generated *de novo* from other cell types provides a new target to control tumor growth and metastasis.

Research Design and Methods

Aim 1) Does PTN induces transdifferentiation of glioma cells?

To test our hypothesis that cell transdifferentiation into endothelial cells contributes to tumor vascularization, we will focus on glioma tumors for two reasons. 1] Gliomas are highly vascularized tumors and an efficient blood supply is of critical importance for their growth. 2]

Recently, PTN was found to be expressed by human glioma cell lines, cell cultures derived from solid gliomas, and glioma sections [Mentlein, 2002 #3219]. PTN mRNA or protein was detected in all WHO III and IV grade gliomas and cells analyzed *in vitro* or in situ. In situ, PTN expression was restricted to distinct parts/cells of the tumor. PTN showed a strong chemotactic effect on murine BV-2 microglial cells [Mentlein, 2002 #3219]. The human *PTN* gene is localized on chromosome 7, band q33-34 [Li, 1992 #3022]. This chromosome is often amplified in gliomas [Sehgal, 1998 #3221], and therefore enhanced PTN expression may result from this malignant transformation.

This aim will expand our preliminary data by asking whether the transdifferentiation activity of PTN is restricted to monocytic cells or PTN can also induce glioma cells to transdifferentiate into endothelial-like cells. We hypothesized that PTN is a plastogenic factor that promotes tumor neovascularization through transdifferentiation of cells into endothelial-like cells. To test this idea, we will express PTN in glioma cells and determine the phenotypic characteristics of the infected cells.

We have already made the retrovirus expressing PTN and the preliminary data show that the retrovirus infects macrophage cells and the infected cells express PTN. We will use an equivalent approach to infect glioma cells. To save space, we will not repeat the methodology to infect glioma cells. We have already received two glioma cell lines U 87 and MO59 that are growing in our laboratory. The exponentially growing cells will be infected with the bicistronic retrovirus harboring GFP, or GFP+PTN sense strand or GFP+PTN anti-sense strand. The infected cells will be separated from uninfected cells by using G418 selection media and FACS. The expression of PTN in the infected cells will be assessed by Northern and Western blot analyses using the probes that we have developed. The expression of endothelial cell markers will be evaluated by RT-PCR and immunostaining as described above. In addition, we will determine the expression of GATA-2, GATA-3, and Oct-4 transcription factors in the uninfected and infected cells.

Outcome:

We do not anticipate any major technical hurdles in carrying out the experiments. We have the expertise and the reagents necessary to perform the experiments.

Previous studies have shown that glioma cells express PTN. If, PTN converts glioma cells into endothelial cells, we anticipate that the uninfected glioma cells will express endothelial

cell markers. To our knowledge, there has been any report about the expression of endothelial cell markers in glioma cells.

It is possible that we do not detect expression of endothelial cell markers in uninfected cells, whereas glioma cells infected with PTN sense strand express endothelial cell markers. One explanation for such a finding would be gene dose effect. We do not know the level of PTN gene expression that is required for the conversion of monocytic cells into macrophages. In addition, we do not know whether the level of endogenous PTN produced by glioma cells would be sufficient for conversion of cells into endothelial cells. In addition to tumor cells, macrophages are a major component of the leukocyte infiltrate of tumors [Balkwill, 2001 #3258]. Tumor-associated macrophages have complex dual functions in their interaction with neoplastic cells, and evidence suggests that they are part of inflammatory circuits that promote tumor progression [Balkwill, 2001 #3258]. Our preliminary data showed that activated macrophages express PTN. Thus, in tumors, there appear to be two sources of PTN: tumor cells and macrophages. It is possible that the level of endogenous PTN produced by tumor cells *per se* may not be sufficient for the conversion of tumor cells into endothelial cells. Additional source of PTN, such as macrophages, may be required to generate enough of a dose of PTN necessary for transdifferentiation to occur. Therefore, the uninfected glioma cells may not express endothelial cell markers whereas cells infected with PTN sense strand express sufficient dose of PTN required for transdifferentiation processes to occur.

Previously, Choudhuri *et al.* [Choudhuri, 1997 #3193] overexpressed PTN in MCF-7 cells, a breast carcinoma cell line. Although the authors did not investigate the phenotypic modulation of cells by PTN, they did showed that PTN-infected MCF-7 cells produced tumors that grew significantly faster than uninfected cells or cells transfected with a control DNA plasmid. Furthermore, these PTN-induced tumors had a greater vascular density compared to control tumors. In a corneal angiogenesis assay, it was shown that corneas receiving PTN-infected MCF-7 cells scored a higher angiogenic response when compared to using uninfected cells. It was found that the vascular pattern in the PTN-expressing tumors was strikingly different from those expressing VEGF. While MCF-7 cells expressing VEGF elicited an angiogenic response within 48 h [Zhang, 1995 #3196], those expressing PTN required 2 weeks [Choudhuri, 1997 #3193]. We suggest that this relatively long incubation time required to induce angiogenesis by PTN-infected MCF-7 cells may be related to a phenotypic alteration of

MCF-7 cells into endothelial-like cells. This aim will explore this possibility. We anticipate that we will observe similar activity for glioma cells infected with PTN compared to uninfected glioma cells.

Aim 2) What is the active domain of PTN?

This aim will extend the preliminary data by asking which segment or domain of the PTN molecule is responsible for its transdifferentiation activity. We hypothesize that the potential of PTN to induce transdifferentiation is mediated by several functional domains.

Prior studies have demonstrated that PTN is a mitogen and utilized a series of constructed PTN mutant proteins to determine the domains required for the transformation activity [Zhang, 1999 #3187]. It was found that a combination of two PTN segments was required for the transformation activity. However Inui *et al* [Inui, 2000 #3194] showed that only one segment corresponding to the N-terminal domain of PTN was required for transformation activity of PTN. Others have shown that while the C-terminal end of PTN was required for transformation activity, the N-terminal segment retained its neurite outgrowth activity [Bernard-Pierrot, 2001 #3195], suggesting that mitogenic and neurite outgrowth activities are mediated through different pathways. Employing a similar strategy, we will construct a series of PTN mutants and test them for their ability to transdifferentiate THP-1 and RAW cells.

The PTN mutants will be constructed with consideration to (i) the N- and C-terminals which contain heparin-binding β-sheet domains [Kilpelainen, 2000 #3189] and (ii) the middle portion that is a flexible linker between the terminal ends and is associated with transformation activity [Zhang, 1999 #3187]. We have already cloned full-length human PTN. We will design PCR primers corresponding to the N-and C-terminal halves of the protein. Using the full-length PTN as a template, we will amplify the N- and C-terminal domains of PTN and the veracity of nucleotide sequence will be verified by DNA sequencing. The PCR products will be cloned into the TOPO PCR-2 vector (Invitrogen), followed by subcloning into a retroviral bicistronic vector that we have developed (discussed above), using standard molecular biology techniques. As described above, the retroviral vectors will be packaged, viral titers will be determined, and monocytic cells will be infected with the viruses. The expression of endothelial cell markers will be assessed as discussed above using PCR and immunohistochemical staining. Endothelial cell markers (e.g., FLK-1, Tie-2, VE-cadherin, PECAM-1, endothelial nitric oxide synthase and the

von Willebrand factor) will be utilized to determine the extent of transdifferentiation *in vitro*. The ability of infected cells to promote tumor vascularization *in vivo* will be assessed by standard xenograft transplantation experiments using PTN-infected glioma cells or human THP-1 monocytic cells. Infected cells will be injected subcutaneously at a unique site in mice. Tumor size will be measured twice a week, starting from the second week following injection. Mice will be sacrificed 6 weeks after injection.

Outcome:

We do not anticipate major technical problems in carrying out the experiments described above. We have already cloned full-length wild type PTN and the retrovirus that we have generated is functional and capable of infecting cells. The deletion experiments are straightforward.

Previous study has shown that infection of breast cancer cells with a truncated mutant of PTN reverted the transformed phenotype of the breast cancer cells [Zhang, 1997 #3179]. PTN is a heparin binding protein and its N- and C-terminal domains have a strong net positive charge, suggesting they could interact with the receptor or an associated second "low affinity" receptor through electrostatic forces but are unlikely to signal active site-mediated receptor functions. Recently, N-syndecan has been implicated as a PTN binding protein [Raulo, 1994 #3031], however, the binding of N-syndecan to PTN is not specific to PTN since basic fibroblast growth factor (bFGF) competes for PTN binding sites, and the glycosaminoglycan chains alone of N-syndecan bind both PTN and bFGF [Raulo, 1994 #3031]. N-syndecan functions as a low affinity receptor and appears to regulate binding of bFGF to its high affinity receptor [Chernousov, 1993 #3262]. There is no data about the specific receptor that mediates interaction of PTN with either macrophages or glioma cells. In the complete absence of information about the receptor, it is difficult to predict the outcome of experiment. However, our data will provide a structural basis for further studies on the functions of PTN in transdifferentiation in glioma cells and other human tumors. Our findings also will provide a molecular model system to dissect the functional responses in tumors constitutively expressing PTN.

Aim 3) What is the downstream signaling in PTN-mediated transdifferentiation?

This aim will extend the effort of aim 2 by investigating the PTN-induced signaling in monocytes/macrophages. Currently, nothing is known about PTN signaling in monocytes/macrophages. As an initial step to understand PTN signaling involved in

transdifferentiation of monocytes/macrophages to endothelial cells, we will concentrate our effort on the mitogen-activate protein (MAP) kinase pathway for two reasons: 1] this pathway is known to be activated by PTN in bovine epithelial lens cells [Souttou, 1997 #3086], and 2] MAP kinase pathway is thought to be a key signaling pathway that have been implicated in the phenotypic outcome for endothelial cells and angiogenesis [Lee, 2000 #3213]. Activation of MAP kinase pathway has been investigated in a variety of macrophages including THP-1 [McGilvray, 1998 #3198;Hambleton, 1996 #3199;Huang, 1999 #3200;Kurosawa, 2000 #3201;Bowling, 1996 #3202] and RAW [Hambleton, 1996 #3203;Chen, 1999 #3204;Ajizian, 1999 #3205;Petrache, 1999 #3206] cells. We will use inhibitors of MAP kinase to block the transdifferentiation of THP-1 and RAW cells transduced with PTN retroviruses. Control cells will include uninfected cells and cells infected with retroviruses carrying only the GFP reporter gene. After infection with the retroviruses, cells will be allowed to establish an efficient level of transduction for 24-48 hours. Cells will then be treated with inhibitors of MAP kinase which known to be effective in THP-1 and RAW cells such as SB203580 [Lee, 1994 #3208] (Calbiochem, San Diego, CA). After treating for various time points, the expression of endothelial cell markers will be assessed, as we described above. To confirm the activation of the MAP kinase signaling pathway in cells undergoing transdifferentiation, we will perform established Western blot analysis to assess phosphorylation of specific protein substrate targets (e.g. SHC, ERK1, ERK2, and Akt1) in the PTN-transduced cells. Control cells that are not undergoing transdifferentiation should display lower levels of these phosphorylated proteins.

Outcome:

We predict that the MAP kinase inhibitors will block the transdifferentiation activity of PTN, suggesting that this pathway is involved in PTN-mediated transdifferentiation of monocytes/macrophages into endothelial-like cells. Alternatively, it is possible that the transduced THP-1 and RAW cells have already committed to the endothelial cell phenotype during the initial period of retroviral transduction and therefore, the inhibitors remain ineffective. To address this issue, we will add the inhibitors just prior to retrovirus infection in an attempt to block the transdifferentiation from proceeding. It is also possible that other pathways are responsible for PTN-induced signaling in monocyte/macrophages. As an initial step to explore such possibility, we will focus on the activation of Src which as been postulated as an intermediary signaling molecule for PTN activity [Muramatsu, 2002 #3021].

F. VERTEBRATE ANIMALS

Proposed animal use. We will use standard xenograft transplantation experiments for *in vivo* studies. Infected cells will be injected subcutaneously at a unique site in nude mice (Jackson Laboratories). Tumor size will be measured twice a week, starting from the second week following injection. Mice will be sacrificed 6 weeks after injection.

Justification for animal use. Xenografts of cancer cells into nude mice have been widely used to investigate tumor vascularization *in vivo*.

Veterinary care. All animals will be maintained at the Burns and Allen Research Institute of the Cedars-Sinai Medical Center. The vivarium at this institution is a registered research facility with the United States Department of Agriculture and is fully accredited by the American Association of Accreditation of Laboratory Animal Care (AAALAC).

Procedures for minimizing pain, discomfort and stress. For transplantation experiment, mice will be anesthetized intraperitoneally with injection of ketamine and xylazine. Post-survival surgery, mice will be given buprenorphine 0.05-0.1 mg/kg SQ.BID (q 12 hr) for a minimum of two doses for analgesia. For euthanasia, mice will be euthanized either by (i) carbon dioxide inhalation followed by decapitation or (ii) decapitation while under anesthesia (ketamine and xylazine).

Euthanasia methodology. Animals will be terminated consistent with recommendations of the Panel on Euthanasia of the American Veterinary Medical Association.

WHAT IS CLAIMED IS:

1. A method of inhibiting neovascularization, comprising:

providing a compound that inhibits the activity of pleiotrophin (PTN) or its effects; and

administering the compound to a mammal.

2. A method of promoting neovascularization, comprising:

providing a compound that enhances or promotes the activity of pleiotrophin (PTN); and

administering the compound to a mammal.

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